(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/78969 A1

(51) International Patent Classification[7]: C12N 15/49, C07K 14/16, A61K 38/10, 39/02, C07K 5/037, 14/445

(21) International Application Number: PCT/US00/10119

(22) International Filing Date: 12 April 2000 (12.04.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/338,318 21 June 1999 (21.06.1999) US
60/171,742 22 December 1999 (22.12.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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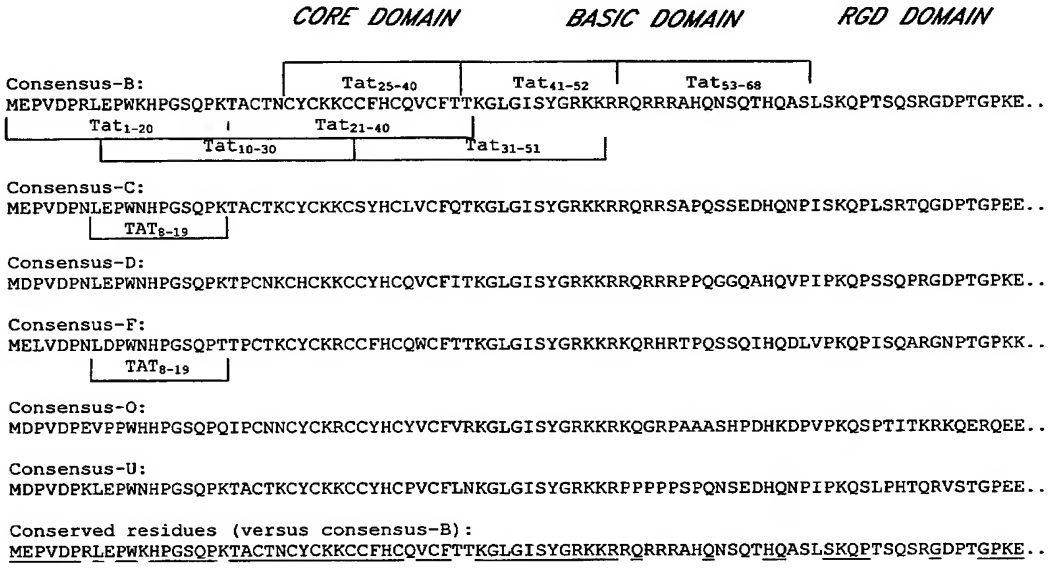
(54) Title: HIV TAT PEPTIDES AND MULTIPLE PEPTIDE CONJUGATE SYSTEM



(57) Abstract: The present invention relates to pathogenic HIV-1-Tat peptides, multiple peptide conjugates, and HIV-1-Tat-multiple peptide conjugate, methods of preparing them, and methods of using them to induce an immune response.

WO 00/78969 A1

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

HIV TAT PEPTIDES AND MULTIPLE PEPTIDE CONJUGATE SYSTEM

PART I: PATHOGENIC HIV-1-TAT PEPTIDES

Background of the Invention

Infection with HIV, the causative agent of Acquired Immune Deficiency Syndrome (AIDS), is responsible for a large number of deaths annually and represents a significant threat to human health. Accordingly, an extensive effort has been mounted to characterize the HIV virus and to identify potential targets for therapeutics.

The HIV Tat protein, a transactivator which regulates gene expression from the HIV long terminal repeat (LTR) represents one attractive therapeutic target. The Tat protein of human immunodeficiency virus type-1 (HIV-1) is an 86 amino acid protein released by infected cells and plays a critical role in the progression of HIV disease (Jeang, *J. Biomed. Sci.* 5: 24 (1998); Ensoli *et al.*, *J. Virol.* 67: 277(1993)). Transactivation of the HIV-LTR promoter by the Tat protein is essential for both viral gene expression and virus replication. Extracellular Tat released by infected cells during the acute phase of infection enters non-infected cells and disrupts many host immune functions by activating a wide variety of genes regulated by specific viral and endogeneous cellular promoters (Vaishnav and Wong-Staal, *Ann. Rev. Biochem.* 60: 577 (1991); Kumar *et al.*, *J. Immunol.* 161: 776 (1998))

In addition, Tat mimics many of the effects of HIV infection on monocytes including increased MMP-9 and cytokine production, and collagen expression in glioblastoma cells (Lafrenie *et al.*, *J. Immunol.* 159: 4077 (1997); Lafrenie *et al.*, *J. Immunol.* 156: 1638 (1996); Taylar *et al.*, *Proc. Natl. Acad. Sci., USA* 89:9617 (1992)) These observations correlate with the high levels of cytokines such as IL-1, IL-6, and TNF found in sera from HIV-infected individuals that lead to an increase in the level of HIV replication, thus suggesting that extracellular Tat promotes the intracellular pathogenic process. However, Tat has multiple domains, and consequently how Tat induces these diverse effects is not clearly understood.

Summary of the Invention

HIV-1 encodes the transactivating protein Tat, which is essential for virus replication and progression of HIV disease. However, Tat has multiple domains, and consequently the molecular mechanisms by which it acts remain unclear. We show that cellular activation by Tat involves a short core domain, Tat_{21-40}, containing only 20 amino acids including seven cysteine residues highly conserved in most HIV-1 subtypes. Effective induction by Tat_{21-40} of both NF-κB-mediated HIV replication and TAR-dependent transactivation of HIV-long terminal repeat indicates that this short sequence is sufficient to promote HIV infection. Moreover, Tat_{21-40} possesses potent angiogenic activity, further underscoring its role in HIV pathogenesis. These data provide the first demonstration that a 20-residue core domain sequence of Tat is sufficient to transactivate, induce HIV replication, and trigger angiogenesis. This short peptide sequence provides a novel therapeutic target for disrupting the functions of Tat and inhibiting progression of HIV disease.

Brief Description of the Drawings

Figure 1. Amino acid sequence of HIV-Tat protein from various subtypes, designated consensus B (SEQ ID NO: 74), consensus C (SEQ ID NO: 75), consensus-D (SEQ ID NO:76), consensus-F (SEQ ID NO:77), consensus-O (SEQ ID NO:81); and consensus-U (SEQ ID NO:79). Highly conserved residues are indicated at the bottom (SEQ ID NO:80).

Figure 2 shows the chemical structure of one embodiment of a core template of the present invention.

Figure 3 shows the chemical structure of one embodiment of a base peptide of the present invention.

Figure 4 shows the chemical structure of one embodiment of a multiple peptide conjugate of the present invention.

Figure 5A and B show the sequences of malarial peptides and HIV-1 peptides used in some embodiments of the multiple peptide conjugates of the present invention. (A) Letters underlined represent Hmb derivatized amino acids for malaria peptides; (B) letters underlined show the position of the seven cysteine(tButhio) residues in the HIV-Tat peptide (HIV-1-Tat-1).

Figure 6 illustrates one embodiment of the synthetic process which was used to prepare a multiple peptide conjugate.

Figure 7A illustrates one embodiment of a core template of the present invention.

Figure 7B illustrates one embodiment of a base peptide of the present invention.

Figure 7C illustrates one embodiment of a multiple peptide conjugate of the present invention.

FIGURE 8. Chemical structure of HIV-1-Tat-MPC comprising functional epitopes of the HIV-1-Tat protein.

FIGURE 9. Effect of HIV-1-Tat synthetic peptide on HIV replication in monocytes. Monocytes were infected with HIV_{Ba-L} in the presence or absence of recombinant HIV-1 Tat or the group O peptide. At day 6, culture supernatants were harvested, and cell-free virus was determined by p24 ELISA. The data are representative of two separate experiments.

FIGURE 10. Panel *a*, Humoral response in mice immunized with various doses (10 ug, 20 ug, and 100 ug) of the HIV-1-Tat-MPC. Antisera were assayed by ELISA on 96-well plates coated with HIV-1-Tat-MPC at a concentration of 1 ug/ml. Titers shown as OD values at 650 nm of 1000-fold diluted serum specimens. Panel *b*, Anti-HIV-1-Tat-MPC titer of sera at various dilutions, where reactivity was determined as described for panel *a*. (•) 10 ug HIV-1-Tat-MPC; (o) 20 ug HIV-1-Tat-MPC; ( ) 100 ug HIV-1-Tat-MPC.

FIGURE 11. Panel *a*, Binding of anti-HIV-1-Tat-MPC antisera to recombinant (rTat) protein and Tat peptides. Ninety-six (96) well plates were coated with 10 ug/ml of rTat protein or Tat peptides as indicated and blocked with SuperBlock blocking buffer in PBS (Pierce). Bound antibodies were detected by ELISA. Panel *b*, Specificity of HIV-1-Tat-MPC antisera to rTat was evaluated by competition experiments using soluble peptides. The ability of purified peptides to inhibit binding of anti-HIV-1-Tat-MPC antibodies to rTat protein was evaluated in the presence of the indicated peptide combinations. Each peptide (10 ug/ml) was incubated with 1:100 diluted antisera prior to addition

onto rTat-coated plates, and bound antibodies were determined by ELISA. Data for triplicate experiments are shown as OD±SEM at 450 nm.

FIGURE 12. Lymphoproliferative responses of spleen cells from Balb/c mice vaccinated with HIV-1-Tat-MPC. Splenocytes (2 x 10^5) prepared from mice immunized with 20 ug of HIV-1-Tat-MPC were incubated with 10 ug rTat , HIV-1-Tat-MPC, or Tat peptides for 96 h at 37°C, and antigen-induced proliferative responses were determined. The bars represent ^{3}H-thymidine incorporation, expressed as cpm±SEM from triplicate cultures of pooled spleen cells from two independent experiments. rTat, $P < 0.005$; HIV-1-Tat-MPC, $P < 0.01$; Tat_{9-20}, $P < 0.001$; Tat_{21-40}, $P < 0.02$; Tat_{53-68}, $P < 0.005$; Tat_{41-52}, $P = 0.97$ versus the control with medium alone.

FIGURE 13. Model illustrating a possible mechanism for inhibition of Tat-induced HIV pathogenesis by anti-HIV-1-Tat-MPC antibodies.

Detailed Description of the Preferred Embodiment

The present invention relates to the identification of functional domains of the HIV Tat protein which mediate viral activation. As will be familiar to those skilled in the art, there are a variety of subtypes of HIV, including the B, C, D, F, M, O, and U subtypes. The present invention uses particular peptide sequences to identify functional domains in Tat. However, it will be appreciated that the particular sequences used in the examples below may be substituted with the corresponding sequences derived from consensus sequences for each subtype, corresponding sequences from individual isolates, or corresponding sequences from subtypes other than those specified above. Accordingly, the present invention specifically contemplates the use of the corresponding sequences from such consensus sequences, isolates or other subgroups.

As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. For example, purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated as falling within the definition of "purified."

As used herein, the term "isolated" requires that the material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated.

Figure 1 lists the consensus sequences of the Tat genes from the B, C, D, F, O, and U subtypes as well as a consensus sequence derived from the sequences from all of these subtypes.

In order to identify Tat-specific sequences responsible for cellular dysfunction, overlapping peptides from various domains of the consensus-B and other HIV-1 subtypes were synthesized. The ability of the peptides to direct HIV replication in monocytes was compared to that of full length recombinant Tat protein as described in Example 1 below.

Example 1

Evaluation of Effects of Peptides on HIV Replication in Monocytes

Tat peptides to be tested for their effects on HIV replication in monocytes were synthesized by solid phase synthesis on an Applied Biosystems Peptide synthesizer Model 430A (Foster City, CA) (Merrifield, *J. Am. Chem. Soc.* 85: 2149 (1963)). After an initial HPLC purification of the crude cysteine-containing peptides, they were redissolved in 0.1 M Tris acetate buffer, pH 8.3, and air-oxidized overnight. Peptides were then subjected to desalting and purification by reverse-phase HPLC, lyophilized, and stored at -70°C. Peptide identities were confirmed by amino acid compositional analysis to determine specific molar ratios for each residue and plasma desorption mass spectroscopic analysis.

Recombinant full length Tat protein was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergies and Infectious Disease, National Institutes of Health, from Dr. Andrew Rice or Dr. John N. Brady (Lafrenie *et al., J. Immunol.*, 156:1638(1996)). HIV-Tat was dissolved at 10 g/ml in treatment buffer (PBS containing 1 mg/ml BSA, 0.1 mM dithiothreitol) and frozen in aliquots at -80°C. Tat preparations were screened and found to be negative for endotoxin contamination.

Monocytes were isolated from the peripheral blood mononuclear cells of donors seronegative for HIV and hepatitis after leukapheresis and purification by countercurrent centrifugal elutriation (Wahl and Smith In *Current Protocols in Immunology*, J. E. Coligan, A. M. Kruisbeek, and D. H. Margulies, E. M. Shevach, and W. Strober, eds., 2:7.6,1 (1991)). Primary monocytes cultured for 5 days were exposed to HIV-1_{Ba-L}, a monocytotrophic HIV strain (Advanced Biotechnologies Inc.), at a multiplicity of infection of 0.01 infectious virus particles/target cell (Dhawan *et al., J. Immunol.* 154: 422 (1995)).

Monocytes cultured for 5 days were treated with recombinant full length Tat protein or test peptides. On day 5, the cells were harvested and the concentration of p24 gag protein (of HIV) in culture supernatants was determined using a DuPont (Wilmington, DE) p24 ELISA test kit. Data representative of two separate experiments are shown in Table I and expressed as mean +/- SEM of triplicate determinations.

As shown in Table I below, like recombinant full length Tat (rTat), the 20-amino acid core domain Tat_{21-40} containing 7 cysteine residues, all of which are strongly conserved in various subtypes, enhanced HIV replication by greater than four-fold. A peptide derived from the basic domain (Tat_{53-68}) induced a lesser increase in viral replication compared to Tat_{21-40}. Likewise, the Tat_{9-20} peptide also greatly enhanced HIV replication. In contrast, Tat_{41-52}, a peptide sequence located between the core and the basic domains, and a variety of peptides from other positions in the Tat sequence, had no significant effect on HIV replication.

Table I. *Effect of various Tat peptides on HIV replication in monocytes*

Residue numbers	Tat sequence	p24 (pg/ml) (Mean ± SEM)
Control		471 ± 10
T_{1-86}	rTat (recombinant full-length Tat)	1886 ± 26

Tat_{1-20}	MEPVDPRLEPWKHPGSQPKT[1]	421 ± 3
Tat_{10-30}	PWKHPGSQPKTACTNCYCKKC[2]	378 ± 4
Tat_{21-40}	ACTNCYCKKCCFHCQVCFTT[3]	1958 ± 101
Tat_{31-51}	CFHCQVCFTTKGLGISYGRK[4]	429 ± 24
Tat_{53-68}	RQRRRAHQNSQTHQAS[5]	969 ± 75
Tat_{25-40}	CYCKKCCFHCQVCFTT[6]	836 ± 123
Tat_{8-19}	LDPWNHPGSQPT[7]	370 ± 2
Tat_{41-52}	KGLGISYGRKKR[8]	569 ± 9
Tat_{8-19}	LEPWNHPGSQPK[9]	376 ± 2
Tat_{9-20}	PPWHHPGSQPQI[10]	1513 ± 12

The cytopathic effects of the Tat peptides were examined in monocytes as described in Example 2.

Example 2

Monocytes were infected with HIV and contacted with recombinant full length Tat or Tat peptides as described above. At five days postinoculation, cells were washed once with PBS, fixed, and Wright-stained. HIV-associated cytopathic effects were determined by examining the formation of multinucleated giant cells.

Consistent with its enhancement of viral replication, Tat_{21-40} treatment produced a marked increase in HIV-associated cytopathic effects in monocytes as indicated by formation of multinucleated giant cells; the effects were similar to those induced by rTat protein itself. The effect of Tat_{53-68} was less than that of Tat_{21-40}. Tat_{41-52}, the peptide between core and basic domains, and peptides from other Tat domains did not alter HIV-associated cytopathic effects. Thus, a major active site for stimulating HIV replication and monocyte dysfunction can be localized to the 20-residue peptide Tat21-40 and to a lesser extent to Tat53-68 peptide.

[1] SEQ ID NO:67
[2] SEQ ID NO:68
[3] SEQ ID NO:1
[4] SEQ ID NO:69
[5] SEQ ID NO:2
[6] SEQ ID NO:70
[7] SEQ ID NO:71
[8] SEQ ID NO:72
[9] SEQ ID NO:73
[10] SEQ ID NO:3

One of the mechanisms by which HIV-Tat potentiates HIV replication involves transactivation of the HIV-1 LTR via its binding to the TAR sequence along with other cellular factors, resulting in increased viral transcription initiation and elongation (Cujec et al, (1997) *Mol. Cell. Biol.* 17: 1817). In order to characterize further the mechanism of Tat transactivation of the HIV-LTR, CEM lymphoid cells were transfected with wild-type promoter in the presence of various Tat peptides, and the extent of transactivation was determined using CAT assays (Cujec *et al., Mol. Cell. Biol.* 17: 1817 (1997)) as described in Example 3.

Example 3

Transactivation Assays

Cells were electroporated as described in (Kashanchi *et al., Virology* 227: 431 (1997)). CEM cells (12D7) were cultured at a density of 0.5 to 0.8 $x10^6$ cells/ml with daily media additions. Typically, $5x10^6$ cells were electroporated with 5 g of either purified plasmid or Tat protein and 5 g of reporter plasmid. Tat peptides or Tat protein and the reporter HIV LTR-CAT or the TAR mutant HIV TM26 LTR-CAT were mixed with cells and electroporated using a cell porater apparatus (GIBCO/BRL, Gaithersburg, MD). Cell mixtures were electroporated at 800 F, 240V, in RPMI 1640 medium without serum. Following electroporation, cells were plated in 10 ml complete medium, and samples were collected 24 h later for CAT assays.

The Tat_{21-40} peptide produced a nine-fold induction of HIV-LTR, while full-length rTat produced a 25-fold induction. The actual effectiveness of induction by Tat_{21-40} might be greater than observed due to the low solubility of this complex hydrophobic peptide in aqueous buffers. In contrast, buffer, Tat_{1-20}, Tat_{53-68}, Tat_{25-40}, and Tat_{41-52} demonstrated virtually no significant induction of HIV-1 LTR.

The presence of Cys_{22} in core domain Tat_{21-40} (and 3 adjacent residues) was critical for viral activation, since deletion of these residues substantially reduced the ability of Tat_{21-40} to activate HIV infection (Tat_{25-40} in Table I). Since rTat activation of the HIV-LTR promoter is required for productive HIV replication (Yankulov and Bentley, *Curr. Biol.* 18: R447 (1998)), the demonstration of induction by the Tat_{21-40} sequence conserved in most HIV-1 subtypes further confirms a functional role of Tat_{21-40} in HIV infection. In contrast, transfection of CEM cells with a TAR mutant (HIV TM26 LTR-CAT) construct in the presence of the same peptides failed to induce HIV-LTR activation, confirming that the HIV-LTR activation by Tat peptides was TAR-specific.

Previous studies have demonstrated that the arginine rich basic domain located between residues 49 and 57 constitutes the TAR-binding activity (Garcia *et al., EMBO J.* 8:765 (1989); Weeks *et al., Science* 249: 1281 (1990); Cordingley *et al., Proc. Natl. Acad. Sci., USA* 87: 8985 (1990); Feng and Holland, *Nature* 334:165 (1988)). Mutation or deletion of the basic domain severely diminishes the ability of Tat to transactivate the LTR. The overlapping peptide(s) from this region tested in the present study were not as active as Tat_{21-40} peptide. Thus, the Tat_{21-40} peptide may possess the ability to induce TAR-dependent transcription.

Although the precise mechanism of virus regulation by host factors is not clear, it is generally believed that in addition to other unknown factors, Tat and cytokines play a key role in the pathogenesis of HIV infection. Extracellular HIV-Tat causes activation of intracellular signal transduction pathways that culminate in the production

of various cytokines (Lafrenie *et al., J. Immunol.* 159: 4077 (1997); Chen *et al., J. Biol. Chem.* 272: 22385 (1997)). Therefore, because of its ability to induce host factors, Tat is believed to be a key factor for viral enhancement. HIV-Tat activates both viral and host cell genes, and the host F transcription factor contributes to immune dysregulation during HIV infection (Conant *et al., J Virol.* 70: 1384 (1996); Ott *et al., J. Immunol.* 160: 2872 (1998)). Since macrophages are a well-known reservoir for HIV in vivo, the ability of Tat peptides to activate the expression of F in these cells was examined. Monocytes were treated with rTat and other peptides, nuclear extracts were prepared, and F activity was examined by gel shift assay using an F consensus oligonucleotide as described in Example 4 below.

Example 4

Effects of Tat Peptides on F Activity

Monocytes ($1x10^7$/ml) were treated with rTat protein or Tat peptides at 37°C for 15 min. Nuclear extracts were then prepared and analyzed by gel shift analysis as described in (Dhawan *et al., Eur. J. Immunol.* 27: 2172 (1997)).

The ability of HIV-Tat to activate F was retained in core peptide Tat_{21-40} and to a lesser extent Tat_{53-68}. Treatment of monocytes with the Tat_{21-40} peptide rapidly activated F (within 15 min. after exposure) by greater than 9-fold as compared to 3-fold induction by Tat_{53-68}. Interestingly, despite inducing NF- B activity, Tat_{53-68} had little effect on transactivation of HIV-LTR. These observations delineate two distinct mechanisms for viral activation by HIV-Tat: (a) TAR-dependent transactivation of HIV-LTR involving Tat_{21-40} domain, and (b) TAR-independent activation of virus replication involving the host factor NF- B by an intracellular signal transduction pathway.

The above results are complementary to those recently reported by Mayne *et al.*, who have demonstrated the involvement of protein kinase A, phospholipase C and protein tyrosine kinase in Tat-mediated induction of NF-kB and cytokine production by monocytes (Mayne *et al., Neuroimmunomodulation.* 5:184 (1998)).

Tat is released by HIV-infected cells into the extracellular milieu, and has been implicated as a cofactor in the pathogenesis of Kaposi's sarcoma (Albini *et al., Proc. Natl. Acad. Sci. USA* 92: 4838 (1995)), an angioproliferative disease frequently seen in HIV-infected individuals. There is increasing evidence that HIV-Tat induces endothelial cell migration, invasion, and angiogenic processes in vivo (Albini *et al., Oncogene* 12: 289 (1996)). In order to test for potential angiogenic activity of the core domain implicated above in viral pathogenesis, the ability of Tat peptides to induce neovascularization was examined using the chick chorioallantoic membrane (CAM) assay as described in Example 5 below.

Example 5

Neovascularization Analysis

The chick CAM assay was carried out as described in (Gho and Chae, *J. Biol. Chem.* 272: 24294 (1997)) to determine the angiogenic activity of rTat and its derived peptides. Briefly, salt-free aqueous solution (5 l) containing 5.3 pmol of rTat or its derived peptides (Tat_{21-40}, Tat_{53-68}, or Tat_{41-52}) was loaded onto a 1/4 piece of 15-mm Thermonox disk (Nunc), and the sample was dried under sterile air; control treatments used water only. The disk

loaded with sample was placed on the CAM of a 10 day-old chick embryo. After 72 h incubation, negative or positive responses were scored under a microscope. A positive response was characterized as the appearance of a typical radiating network (spokewheel) pattern of new blood vessels around the loaded samples. Assays for each test sample were carried out in 2 sets of eggs, and each set contained 12 to 15 eggs.

The results indicate that picomole quantities (5.2 pmol/egg) of Tat_{21-40} can induce neovascularization. Recombinant Tat (rTat) alone was less effective in inducing an angiogenic response, as reported by others (Albini *et al.*, *Oncogene* 12: 289 (1996)). No significant angiogenic response was observed using vehicle alone or the control peptide Tat_{41-52} containing sequence between the core and basic domains. Interestingly, Tat_{53-68} from the Tat basic domain also had substantial activity; as noted above, this peptide had either partial or minimal activities in assays for HIV replication, cytopathic effects, and transactivation of the HIV-LTR promoter. The exact mechanism of neovascularization in vivo is not clear. However, one scenario is that Tat-induced cytokines stimulate endothelial cells, degrade basement membrane matrix by local enhancement of MMP-9 secretion, and migrate into adjacent tissue to form new blood vessel networks.

Detectable levels of Tat have been reported in HIV-infected individuals (Westendorp *et al.*, *Nature* 375:497 (1995)), suggesting the presence of extracellular HIV-Tat protein in certain phases of HIV infection. It has also been shown that high levels of anti-Tat antibodies are directly related to low viral load (Re *et al.*, *Clin. Diagn. Lab. Immunol.* 3: 230 (1996); Poznansky *et al.*, *Hum. Gene Ther.* 9: 487 (1998)) in seropositive non-progressor patients. Therefore, a strategy targeting a required site(s) in Tat may be used to provide a novel therapeutic modality to reduce disease progression in HIV-infected individuals.

The above results demonstrate that short core domains of the Tat protein retain activities characteristic of the full length Tat protein. In particular, the Tat_{21-40}, Tat_{53-68} and Tat_{9-20} peptides retain activity. Notably, the Tat_{21-40} peptide, which consists of 7 cysteine residues and only 13 other amino acids, is a potent inducer of HIV transactivation and replication. This domain is highly conserved in various HIV-1 subtypes, including the newly discovered group O. Some of these results are complementary to those demonstrating the involvement of Tat and the core domain in the process of monocyte chemotaxis in response to Tat (Lafrenie *et al.*, *J. Immunol.* 157: 974 (1996); Albini *et al.*, *J. Biol. Chem.* 273: 15895 (1998)), which may contribute to altered immunoregulation in HIV-infected individuals. It is important to note that monocytes differentiate into tissue-resident macrophages, which are non-recirculating cells. HIV-infected macrophages could, therefore, continue to infect neighboring normal cells and contribute to the tissue damage typically seen after HIV infection.

Accordingly, the invention provides the active domain Tat_{21-40}, Tat_{53-68} and Tat_{9-20}, singly or in combination, as a novel therapeutic vaccine or a dominant-negative strategy to reduce Tat-mediated progression of disease in individuals with HIV infection.

As used herein the terminology "peptide comprising a Tat functional domain" refers to fragments of the Tat protein comprising one or more of the Tat_{21-40}, Tat_{53-68} or Tat_{9-20} functional domains from any subtype or isolate of HIV and are not limited to functional domains having the exact sequences used in the experiments described above. In

particular, the terminology "peptide comprising a Tat functional domain" encompasses fragments of the HIV Tat protein comprising the sequence ACTNCYCKKCCFHCQVCFTT (SEQ ID NO: 1), the sequence RQRRRAHQNSQTHQAS (SEQ ID NO: 2), or the sequence PPWHHPGSQPQI (SEQ ID NO: 3). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the B subgroup of HIV, *i.e.* EPWKHPGSQPKT (SEQ ID NO: 4), ACTNCYCKKCCFHCQVCFTT (SEQ ID NO: 5), or RQRRRAHQNSQTHQAS (SEQ ID NO: 6). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the C subgroup of HIV, *i.e.* EPWNHPGSQPKT (SEQ ID NO: 7), ACTKCYCKKCSYHCLVCFQT (SEQ ID NO: 8), or RQRRSAPQSSEDHQNP (SEQ ID NO: 9). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the D subgroup of HIV, *i.e.* EPWNHPGSQPKT (SEQ ID NO: 10), PCNKCHCKKCCYHCQVCFIT (SEQ ID NO: 11), or RQRRRPPQGGQAHQVP (SEQ ID NO: 12). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the F subgroup of HIV *i.e.* DPWNHPGSQPTT (SEQ ID NO: 13), PCTKCYCKRCCFHCQWCFTT (SEQ ID NO: 14), or KQRHRTPQSSQIHQDL (SEQ ID NO: 15). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the O subgroup of HIV *i.e.* PPWHHPGSQPQI (SEQ ID NO: 16), PCNNCYCKRCCYHCYVCFVR (ID NO: 17), or KQGRPAAASHPDHKDP (SEQ ID NO: 18). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from the consensus sequence for the U subgroup of HIV *i.e.* EPWNHPGSQPKT (SEQ ID NO: 19), ACTKCYCKKCCYHCPVCFLN (SEQ ID NO: 20), or PPPPPSPQNSEDHQNP (SEQ ID NO: 21). The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the HIV Tat protein comprising the corresponding sequences from an individual isolate falling within the B, C, D, F, M, O, and U subgroups or fragments of the HIV Tat protein comprising the corresponding sequences from other subgroups.

The terminology "peptide comprising a Tat functional domain" also encompasses peptides that retain at least one activity of the full length Tat protein and which have at least about 60, 70, 80, 90, 95, or 99 % of their amino acid sequence identical to that of SEQ ID NO:1, SEQ ID NO: 2, or SEQ ID NO: 3 as determined by FASTA or BLAST using default opening and gap penalties and a PAM scoring matrix. Percent sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA, two polypeptides are aligned for optimal matching of their respective amino acids (either along the full length of one or both sequences, or along a predetermined portion of one or both sequences). The programs provide a "default" opening penalty and a "default" gap penalty, and a scoring matrix such as PAM 250 (a standard scoring matrix; see Dayhoff *et al.*, in: *Atlas of Protein Sequence and Structure*, vol. 5, supp.3 (1978) can be used in conjunction with the computer program. The percent identity can then be calculated as the total number of identical matches/ length of the sequence within the matched span + number of

gaps introduced into the longer sequence in order to align the two segments. Peptides that are at least 70 percent identical will typically have one or more amino acid substitutions, deletions, and/or insertions. Usually, the substitutions will be conservative so as to have little or no effect on the overall net charge, polarity, or hydrophobicity of the peptide but optionally may increase or decrease the activity of the peptide.

The terminology "peptide comprising a Tat functional domain" also encompasses fragments of the Tat protein which include one or more amino acids adjacent to the Tat_{21-40}, Tat_{53-68} or Tat_{9-20} functional domains. For example, the fragments may comprise about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 70, or more than 70 amino acids adjacent to the functional domains, provided that such additional sequences are consistent with the length of the Tat protein and that the fragments are shorter than the intact Tat protein.

The terminology "peptide comprising a Tat functional domain" also includes peptide analogs or peptidomimetics based on the Tat_{21-40}, Tat_{53-68} or Tat_{9-20} functional domains. Peptidomimetics are molecules that have the same effect as a peptide but are not peptides. Peptidomimetics may be resistant to proteases or cheaper to make than peptides.

The peptide comprising a Tat functional domain may be administered via any of the routes traditionally used to administer compositions which induce an immune response, including intraperitoneal administration, intramuscular administration, intradermal administration, intravascular administration or nasal administration.

If desired, the peptide comprising a Tat functional domain may be administered along with an adjuvant to enhance the level of the immune response. A variety of adjuvants suitable for use in compositions for inducing an immune response are familiar to those skilled in the art, including aluminums like hydroxide and phosphate.

The peptide comprising a Tat functional domain may be administered in a physiologically acceptable carrier. A variety of carriers suitable for use in compositions for inducing an immune response are familiar to those skilled in the art. Such carriers are nontoxic to recipients at the dosages and concentrations employed. For example, the carriers may contain saline, buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrins or chelating agents such as EDTA, glutathione or other stabilizers and excipients.

The peptides comprising a Tat functional domain are administered at a dosage and frequency sufficient to induce a desired level of immune response. Preferably, the level of immune response is sufficient to protect an uninfected individual from infection or to reduce or eliminate the symptoms of HIV infection in an infected individual. It will be appreciated that the dosage and frequency of peptide administration will vary depending on the age, weight, and condition of the individual. For example, the dosage sufficient to induce a desired immune response may range from about 1 μg-100 mg or more, more preferably from about 100μg-100mg or more. However, other dosages may also be used.

In some embodiments, peptides comprising different functional domains of the Tat protein may be administered to the individual. For example, a peptide comprising the Tat_{21-40} domain and a peptide comprising the

Tat_{53-68} domain may be administered to the individual. It will be appreciated that any combination of peptides comprising the Tat_{21-40}, Tat_{9-20}, or Tat_{53-68} may be administered to the individual. The peptides comprising different functional domains of the Tat protein may be administered to the individual simultaneously or at different times.

Alternatively, rather than administering one or more peptides comprising Tat functional domains to an individual, nucleic acids encoding the one or more peptides comprising Tat functional domains may be administered to the individual. The nucleic acids may comprise the nucleic acid sequence from an HIV isolate or subtype consensus sequences or the nucleic acid may be designed to encode the peptide comprising a Tat functional domain using the genetic code.

In this embodiment, a vector comprising a nucleic acid encoding a peptide comprising a Tat functional domain operably linked to a promoter is administered to the individual. The vector may be any of the expression vectors familiar to those skilled in the art, including viral or retroviral vectors. Viral vectors suitable for use in the present invention include adenovirus, adeno-associated virus, herpes virus, and vaccinia virus. Retroviral vectors suitable for use in the present invention include Moloney murine leukemia virus vectors, Harvey murine sarcoma virus vectors, mouse mammary tumor virus vectors, and Rous sarcoma virus vectors. Other vectors that induce a minimal host response to the vector itself may also be used.

The vector may be directly introduced into the individual such that the encoded peptide comprising a Tat functional domain is expressed in the individual. In another approach, the expression vector may be introduced into cells *in vitro*. Cells containing the expression vector are thereafter selected and introduced into the individual, where they express the encoded peptide comprising a Tat functional domain.

Alternatively, the nucleic acid encoding a peptide comprising the Tat functional domain may be administered as naked DNA. Techniques for administering naked DNA are described in U.S. Patent No. 5,580,859. If desired, the nucleic acid may be administered along with bupivacaine.

The nucleic acid encoding a peptide comprising a Tat functional domain may be administered at a dosage and frequency sufficient to induce a desired level of immune response in the individual. Preferably, the level of immune response is sufficient to protect an uninfected individual from infection or to reduce or eliminate the symptoms of HIV infection in an infected individual. It will be appreciated that the dosage and frequency of peptide administration will vary depending on the age, weight, and condition of the individual. For example, the dosage sufficient to induce a desired immune response may range from about 1μg-100mg or more, more preferably from about 100μg-200μg or more. However, other dosages may also be used.

Another aspect of the invention relates to dominant-negative mutants of peptides comprising one or more Tat functional domains. Dominant-negative mutants are mutants which are capable of reducing the level of activity of a wild type Tat protein. Such dominant-negative mutants may be generated by mutagenizing a nucleic acid encoding a peptide comprising a Tat functional domain using techniques familiar to those skilled in the art, including site directed mutagenesis, chemical mutagenesis, or PCR based methods. Alternatively, such dominant-negative mutations may be generated by synthesizing peptides having the desired sequence *in vitro*.

Preferably, the dominant-negative mutant comprises a substitution of one or more amino acids in the peptide comprising a Tat functional domain with another amino acid. For example, where the peptide comprising the Tat functional domain has the sequence ACTNCYCKKCCFHCQVCFTT (SEQ ID NO:1), the A (Alanine) at position 1 of the peptide may be substituted with an amino acid selected from the group consisting of cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In another embodiment, the C (Cysteine) at position 2 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In yet another embodiment, the T (threonine) at position 3 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine. In another embodiment, the N (asparagine) at position 4 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, thereonine, valine, tryptophan, and tyrosine. Alternatively, the C (Cysteine) at position 5 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In still another embodiment, the Y (tyrosine) at position 6 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, and tryptophan. In a further embodiment, the C (Cysteine) at position 7 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In yet another embodiment, the K (lysine) at position 8 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In a further embodiment, the K (lysine) at position 9 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In yet another embodiment, the C (Cysteine) at position 10 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In another embodiment, the C (Cysteine) at position 11 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine,

threonine, valine, tryptophan, and tyrosine. In a further embodiment, the F (phenylalanine) at position 12 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In a further embodiment, the H (histidine) at position 13 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In still a further embodiment, the C (Cysteine) at position 14 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In another embodiment, the Q (glutamine) at position 15 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, serine, threonine, valine, tryptophan, and tyrosine. In a further embodiment, the V (valine) at position 16 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, tryptophan, and tyrosine. In another embodiment, the C (Cysteine) at position 17 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In another embodiment, the F (phenylalanine) at position 18 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine. In yet another embodiment, the T (threonine) at position 19 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine. In yet another embodiment, the T (threonine) at position 20 of the peptide may be substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine.

It will be appreciated that similar single amino acid substitutions may also be made in any of the peptides comprising a Tat functional domain, including the particular Tat_{21-40}, Tat_{53-68}, and Tat_{9-20} sequences described above.

The invention contemplates amino, carboxy and internal truncations in a peptide comprising a Tat functional domain. Examples of amino terminal truncations in one of the Tat_{21-40} sequences are provided in Table II below. However, it will be appreciated that amino terminal truncations may also be made in the Tat_{53-68} and Tat_{9-20} sequences or in Tat_{21-40} sequences from other isolates or subtypes.

The truncated peptides can be produced by any of a number of protocols well known to those of skill in the art. Some short examples include, purifying one or more of the peptides taught by the invention, followed by modification of the peptide. Alternatively, the peptides can be produced recombinantly, using molecular biology and biochemical techniques, or synthetically. These peptides may themselves be chemically modified.

Table II

Amino Terminal Truncations

X-FTT-Z (No SEQ ID NO)

X-CFTT-Z (SEQ ID NO: 37)

X-VCFTT-Z (SEQ ID NO: 36)

X-QVCFTT-Z (SEQ ID NO: 35)

X-CQVCFTT-Z (SEQ ID NO: 34)

X-HCQVCFTT-Z (SEQ ID NO: 33)

X-FHCQVCFTT-Z (SEQ ID NO: 32)

X-CFHCQVCFTT-Z (SEQ ID NO: 31)

X-CCFHCQVCFTT-Z (SEQ ID NO: 30)

X-KCCFHCQVCFTT-Z (SEQ ID NO: 29)

X-KKCCFHCQVCFTT-Z (SEQ ID NO: 28)

X-CKKCCFHCQVCFTT-Z (SEQ ID NO: 27)

X-YCKKCCFHCQVCFTT-Z (SEQ ID NO: 26)

X-CYCKKCCFHCQVCFTT-Z (SEQ ID NO: 25)

X-NCYCKKCCFHCQVCFTT-Z (SEQ ID NO: 24)

X-TNCYCKKCCFHCQVCFTT-Z (SEQ ID NO: 23)

X-CTNCYCKKCCFHCQVCFTT-Z (SEQ ID NO: 22)

X-ACTNCYCKKCCFHCQVCFTT-Z (SEQ ID NO: 1)

"X" may represent an amino group, a hydrophobic group, including but not limited to carbobenzoxyl, dansyl, or T-butyloxycarbonyl; an acetyl group; a 9-fluorenylmethoxy-carbonyl (FMOC) group; a macromolecular carrier group including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates.

"Z" may represent a carboxyl group; an amido group; a T-butyloxycarbonyl group; a macromolecular carrier group including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates.

Examples of carboxy-terminal truncations in one of the Tat_{21-40} sequences are provided in Table III below. However, it will be appreciated that carboxy terminal truncations may also be made in the Tat_{53-68} and Tat_{9-20} sequences or in Tat_{21-40} sequences from other isolates or subtypes.

Table III

Carboxy-terminal Truncations

X-ACT-Z (No SEQ ID NO)

X-ACTN-Z (SEQ ID NO: 53)

X-ACTNC-Z (SEQ ID NO: 52)

X-ACTNCY-Z (SEQ ID NO: 51)

X-ACTNCYC-Z (SEQ ID NO: 50)

X-ACTNCYCK-Z (SEQ ID NO: 49)

X-ACTNCYCKK-Z (SEQ ID NO: 48)

X-ACTNCYCKKC-Z (SEQ ID NO: 47)

X-ACTNCYCKKCC-Z (SEQ ID NO: 46)

X-ACTNCYCKKCCF-Z (SEQ ID NO: 45)

X-ACTNCYCKKCCFH-Z (SEQ ID NO: 44)

X-ACTNCYCKKCCFHC-Z (SEQ ID NO: 43)

X-ACTNCYCKKCCFHCQ-Z (SEQ ID NO: 42)

X-ACTNCYCKKCCFHCQV-Z (SEQ ID NO: 41)

X-ACTNCYCKKCCFHCQVC-Z (SEQ ID NO: 40)

X-ACTNCYCKKCCFHCQVCF-Z (SEQ ID NO: 39)

X-ACTNCYCKKCCFHCQVCFT-Z (SEQ ID NO: 38)

X-ACTNCYCKKCCFHCQVCFTT-Z (SEQ ID NO: 1)

"X" may represent an amino group, a hydrophobic group, including but not limited to carbobenzoxyl, dansyl, or T-butyloxycarbonyl; an acetyl group; a 9-fluorenylmethoxy-carbonyl (FMOC) group; a macromolecular carrier group includig but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates.

"Z" may represent a carboxyl group; an amido group; a T-butyloxycarbonyl group; a macromolecular carrier group including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates.

Examples of internal truncations in one of the Tat_{21-40} sequences are provided in below. However, it will be appreciated that internal truncations may also be made in the Tat_{53-68} and Tat_{9-20} sequences or in Tat_{21-40} sequences from other isolates or subtypes.

For example, the internal truncations include

-CTN- and CTN-5-20, wherein 5-20 means C, CY, CYC, ...CYCKKCCFHCQVCFTT- (SEQ ID NO: 54)

-TNC- and TNC-6-20, wherein 6-20 means Y, YC, YCK...YCKKCCFHCQVCFTT- (SEQ ID NO: 55)

-NCY- and NCY-7-20 wherein 7-20 means C, CK, CKK...CKKCCFHCQVCFTT- (SEQ ID NO: 56)

-CYC- and CYC-8-20 wherein 8-20 means K, KK, KKC...KKCCFHCQVCFTT- (SEQ ID NO: 57)

-YCK- and YCK-9-20 wherein 9-20 means K, KC, KCC...KCCFHCQVCFTT- (SEQ ID NO: 58)

-CKK- and CKK-10-20 wherein 10-20 means C, CC, CCF...CCFHCQVCFTT- (SEQ ID NO: 59)

-KKC- and KKC-11-20 wherein 11-20 means C, CF, CFH...CFHCQVCFTT-(SEQ ID NO: 60)

-KCC- and KCC 12-20 wherein 12-20 means F, FH, FHC, ..FHCQVCFTT- (SEQ ID NO: 61)

-CCF- and CCF 13-20 wherein 13-20 means H, HC, HCQ...HCQVCFTT- (SEQ ID NO: 62)

-CFH- and CFH-14-20 wherein 14-20 means C, CQ, CQV...CQVCFTT- (SEQ ID NO: 63)

-FHC- and FHC-15-20 wherein 15-20 means Q, QV, QVC...QVCFTT- (SEQ ID NO: 64)

-HCQ- and HCQ-16-20 wherein 16-20 means V, VC, VCF...VCFTT- (SEQ ID NO: 65)

-CQV- and CQV-17-20 wherein 17-20 means C, CF, CFT...CFTT- (SEQ ID NO: 66)

-QVC- and QVC-18-20 wherein 18-20 means F, FT, or FTT-

-VCF and VCF-19-20 wherein 19-20 means T or TT

CFT and CFTT (SEQ ID NO: 37)

Another type of dominant-negative mutant peptide combines a functional domain peptide that has been inactivated with another peptide that facilitates its binding to the normal target site. One example of such a chimeric dominant negative peptide combines the Tat_{21-40} sequence containing an amino acid substitution that inactivates its function but fails to convert it to an effective dominant negative inhibitor in a peptide chimera with a second peptide sequence such as Tat_{53-68} in order to enhance binding to a molecular target normally bound by both peptide regions in the full length Tat molecule. In this case, the separate peptides may have low or no inhibitory activity, while the composite peptide containing both peptide sequences would have dominant negative activity. A spacer of amino acids between the two peptides, such as repeats of glycine or glycine plus serine, may be added to obtain optimal spacing of functional domains for full activity of the chimera.

A second type of chimeric dominant negative peptide uses a functional peptide domain inactivated by amino acid substitutions containing additional charged amino acids at the ends of the peptide to strengthen its binding to the target site. For example, 4-8 lysine residues added to the amino terminus, the carboxy terminus or both termini will extend the length of the peptide by adding more positive charge in order to increase its binding to negatively charged targets such as DNA.

A third type of chimeric dominant negative peptide combines an inactivated functional domain with some other type of functional binding peptide sequence besides a known Tat peptide or charged amino acids.

The strategy of each of the above types of chimeric peptides is to use a functional Tat peptide domain that has been inactivated by mutation in combination with some other binding moiety that enhances binding of the chimera in order to prevent the binding or function of native Tat molecules.

Dominant-negative mutants produced using the above methods are identified by determining their ability to reduce the activity level of wild type Tat protein. For example, a nucleic acid encoding a prospective dominant-negative mutant may be introduced into a cell containing a gene encoding the wild type full length Tat protein and a Tat-activated reporter gene which encodes a detectable product. In one method, a nucleic acid encoding a prospective dominant-negative mutation is operably linked to the hCMV promoter and cotransfected into BALB/c 3T3 cells with a vector encoding wild type full length Tat and a reporter vector in which the CAT gene is operably linked to the HIV LTR. Control cells are transfected with the vector encoding full length Tat and the CAT reporter vector. Those

mutants which reduce the levels of CAT expression relative to that in the control cells are dominant-negative mutations.

Peptides comprising a Tat functional domain containing a dominant-negative mutation may be used in any of the methods for inducing an immune response described above, including methods in which the peptides or nucleic acids encoding the peptides are administered to an individual. In another embodiment, peptides comprising a Tat functional domain containing a dominant-negative mutation are administered to an individual as described above. The peptides inhibit the activity of the wild type Tat protein in the individual.

Alternatively, nucleic acids encoding peptides comprising a Tat functional domain containing a dominant-negative mutation may be operably linked to a promoter in any of the vectors described above. Preferably, the vector does not induce an immunological response to itself. The vector is introduced into the individual or introduced into cells in vitro which are later introduced into the individual as described above. The peptides comprising a Tat functional domain containing a dominant-negative mutation are expressed in the individual and inhibit the activity of wild type Tat protein in the individual.

Antibodies to a peptide comprising a Tat functional domain are another embodiment of the present invention. The antibodies may be monoclonal antibodies or polyclonal antibodies.

Monoclonal antibody to the peptides comprising a Tat functional domain can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495 (1975)), or derivative methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the peptide over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Meth. Enzymol.* 70:419 (1980)), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. *et al. Basic Methods in Molecular Biology* Elsevier, New York. Section 21-2. If desired, the antibodies can be humanized to reduce the risk of immune reactions against non-human epitopes on the antibody.

Polyclonal antiserum containing antibodies to peptides comprising a Tat functional domain can be prepared by immunizing suitable animals with the peptide, which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable.

An effective immunization protocol for rabbits can be found in Vaitukaitis, J. *et al.* (1971) *J. Clin. Endocrinol. Metab.* 33:988-991.

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined by ELISA or semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. *et al.*, Chap. 19 in: *Handbook of Experimental Immunology* D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 M). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: *Manual of Clinical Immunology*, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980).

Antibody preparations prepared according to either protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample. The antibodies may also be used in therapeutic compositions for reducing the levels of the protein in the body.

In addition to their therapeutic uses, the peptides comprising a Tat functional domain may be used as reagents to study HIV replication and the HIV life cycle. For example, the peptides may be used to identify proteins which interact with the functional domains in systems such as the yeast two hybrid systems such as the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech).

The peptides comprising a Tat functional domain may also be used in affinity chromatography to identify proteins which interact with the functional domains. In such systems, the peptides comprising a Tat functional domain are linked to a suitable chromatography matrix. Samples comprising proteins which bind to the functional domain are placed in contact with the matrix under conditions which allow a complex to be formed between the functional domain and the proteins which bind thereto. After a wash to remove non-specifically bound proteins, specifically bound proteins are eluted. The specifically bound proteins can be sequenced and clones encoding them can be identified using techniques familiar to those skilled in the art.

In addition, the peptides comprising a Tat functional domain may be used to identify agents which disrupt the activity of Tat. For example, in the case of the Tat_{21-40} functional domain, a peptide comprising the Tat_{21-40} functional domain may be bound to the HIV TAR sequences to form a complex. The complex can be contacted with an agent to determine whether the agent disrupts the complex.

Alternatively, the cells containing a peptide comprising a functional domain of the Tat protein can be contacted with the agent to determine whether the agent disrupts at least one activity of the peptide, including such activities as activation of the HIV LTR, angiogenesis or NF-κB activation. The agents tested for disruption of the activity of the peptide comprising the Tat functional domain may include organic or inorganic molecules, biochemical molecules, peptides, or polynucleotides.

Another embodiment of the present invention is a method for determining the disease state of an individual infected with HIV. Non-progressors have a high titer of antibodies against Tat in their sera, while rapid progressors have

low titers of antibodies against Tat in their sera. In this embodiment of the present invention, sera is obtained from an individual. The sera is contacted with a peptide comprising a Tat functional domain, and the amount of antibody in the sera which binds to the peptide is determined to identify the individual as a non-progressor or a rapid progressor. The amount of antibody in the sera which binds to a peptide comprising a Tat functional domain may be determined using conventional methods familiar to those skilled in the art, including ELISA analyses or Western blots. In an ELISA analysis, a peptide comprising a Tat functional domain is attached to a microtiter well and contacted with serum from an individual. The amount of antibody against the peptide is determined by adding a detectably labeled secondary antibody.

In a Western analysis, the peptide comprising a Tat functional domain is attached to a filter and contacted with the serum from the individual. The amount of antibody against the peptide is determined using a detectably labeled secondary antibody.

PART II: MULTIPLE PEPTIDE CONJUGATES

Background of the Invention

Traditional vaccines have been prepared using immunogens such as attenuated versions of pathogenic organisms, inactivated pathogenic organisms or preparations containing antigens from the pathogenic organism. For example, the organisms responsible for pertussis, typhoid, and polio have been inactivated and used as vaccines. Cell wall or polysaccharide preparations obtained from Haemophilus or Meningococcus have also been used (Seid et al., *Glycoconjugate J.*6:489-498 (1989); Frasch, *Clin. Microbiol. Rev.* 2 (Suppl.) 134-138 (1989).

While these approaches have produced highly effective vaccines in some instances, they suffer from a variety of complications. Such complications include the presence of adventitious agents in the vaccine compositions, reversion of the organism used in the vaccine to virulence, variation in the properties of the strains used in the vaccination, and risk of cross-reactivity of antigens in the vaccine with host antigens. In addition, in approaches utilizing inactivated organisms, it may be difficult to completely inactivate the organism.

An alternative strategy to conventional vaccines has been to identify epitopes or immunogens which are responsible for a specific response. Synthetic peptides containing these epitopes or immunogens are then used as vaccines (Mills, *Curr. Opinion in Immunol.* 2:804-814 (1989)). Such synthetic molecules are devoid of many of the risks associated with vaccines derived from pathogenic organisms. In addition, the synthesis of peptides with lipophilic or glycosidic functional groups may improve delivery of the antigenic peptides and aid in targeting the antigenic peptide to antigen presenting cells.

Summary of the Invention

The present invention relates to multiple peptide conjugates, methods of preparing them, and methods of using them to induce an immune response.

Definitions

As used herein, "t-Boc" means tert-butoxycarbonyl.

As used herein, "Maldi-tof" means matrix assisted laser desorption-time of flight.

As used herein, "NMP" means N-methylpyrrolidone.

As used herein, "TFA" means trifluoroacetic acid.

As used herein, "HOBt" means 1-hydroxybenzotriazole.

As used herein, "RP-HPLC" means reverse phase-high performance liquid chromatography.

As used herein, "tbu" means tert-butyl.

As used herein, "Fmoc" means 9-fluorenylmethoxycarbonyl.

As used herein, "DCM" means dichloromethane.

As used herein "Npys" means 3-nitro-2pyridine sulphenyl.

As used herein "Mmt" means 4-methoxytrityl.

As used herein, "TCEP" means triscarboxyethylphosphine.

As used herein, "DCC" means N,N'-dicylohexylcarbodiimide.

As used herein, "Trt" means trityl.

As used herein, "TIS" means triisopropylsilane.

As used herein, "Pmc" means 2,2,5,7,8-pentamethylchroman-6-sulfonyl.

As used herein, "DMSO" means dimethylsulfoxide.

As used herein, "Hmb" means N-[2-hydroxy-4-methoxybenzyl].

As used herein, "HBTU" means 2-[1-H-Benzotriazole-1-yl]-1,13,3-tetramethyluronium hexafluorophosphate.

As used herein, "Otbu" means tert-butyl ester.

As used herein, "DCU" means dicyclohexyl urea.

As used herein, "TNBS" means trinitrobenzene sulfonic acid.

As used herein, "NHS" means N-Hydroxysuccimide.

As used herein, "EDT" means ethanedithiol.

As used herein, "CMC" , means carboxymethylcysteine.

As used herein, "Br_2Ac_2O" means Bromoacetic anhydride.

As used herein, "Cl_2Ac_2O" means Chloroacetic anhydride.

As used herein, "Bu_3P" means tri-n-butylphosphine.

As used herein, "MAP" means multiple antigen peptide.

As used herein, "MPC" means multiple peptide conjugate.

As used herein, "SEC" means size exclusion chromatography.

As used herein, "SPPS" means solid phase peptide synthesis.

As used herein, "ESI-MS" means electrospray ionization mass spectroscopy.

As used herein, "linear peptide" refers to a peptide sequence to be attached to the base peptide.

As used herein, "base peptide" refers to the core peptide to which a peptide has been attached or the core template on which a peptide has been synthesized.

Detailed Description of the Preferred Embodiment

As discussed above, vaccines based on inactivated organisms or materials derived from organisms may suffer from a variety of drawbacks. Accordingly, it is desirable to use synthetic compounds to induce an immune response. However, it is generally thought that the conformational B-cell epitopes involved in neutralization of some organisms would be difficult to mimic though the use of a simple synthetic linear molecule (Sesardic, *J. Med. Microbiol.* 39:241-243 (1993); Arnon and Horwitz, *Curr. Opinion in Immunol.* 4:449-453 (1992)).

One approach to address this issue has been described by Tam and others. In this approach, peptides are linked to a core structure to generate a multiple antigen peptide (MAP) (Nardelli et al.*The MAP system: A flexible and unambiguous vaccine design of branched peptides in vaccine design: The subunit and adjuvant approach* Plenum Press, New York, pp803-819 (1995); Drijfhout et al. *Solid-phase and applications of N-(S-acetylmercaptoacetyl) peptides* 187:349-354 (1990); Nardelli et al, *J. Biol. Chem.* 148:914-920(1992); Nardin et al., *Vaccine* 16:590-600 (1998)). However, the classical solid phase synthesis methodology employed in the traditional MAP is not without difficulty. For example, the classical solid phase synthesis methodology employed in this approach may not yield a sufficient amount of product for use in a vaccine or may yield a heterogenous product which is not amenable to use in a vaccine.

For example, problems may arise as a consequence of microheterogeneity in the synthetic compounds (Drijfhout and Bloemhoff, *Int.J. Peptide Protein Res.* 37:27-32 (1991); Grant et al., *Methods in Enzymology* 289: 395-419(1997)). Aggregation and steric hindrance of the growing peptide chain during solid phase peptide synthesis is thought to be a major source of the microheterogeneity found in traditional multiple antigen peptides produced by the direct synthesis approach (Nardin et al., *Vaccine* 16:590-600 (1998); Drijfhout and Bloemhoff, *Int.J. Peptide Protein Res.* 37:27-32.(1991)). Many of these problems are thought to be sequence related and may possibly be caused by inter and intra-chain hydrogen bonding by the peptide backbone, forming beta sheets or other secondary structures (Kent et al., In: *Innovations and perspectives in solid phase synthesis*, Epton R, ed., Intercept Ltd., Andover, UK, p. 1 (1992)). This may lead to significant steric hindrance, thus reducing the efficiency of the acylation or deprotection reactions or both (Milton et al., *J. Am. Chem. Soc.* 112: 6039-6046(1990)). Some groups have shown that peptide aggregation may occur as early as the fifth residue depending upon the peptide sequence and side chain blocking groups used (Bedford et al., *Int. J. Peptide Protein Res.* 40:300-307 (1992)). In order to reduce the effects of hydrogen bonding, protection of the peptide bonds (i.e. by forming a tertiary nitrogen) has been utilized (Johnson et al., *J. Chem. Soc. Chem. Commun.* 4:369-372 (1993)).

In addition to the factors listed above, the procedures for isolating and purifying the synthetic product resulting from the current methods for preparing multiple peptide conjugates are extremely laborious. Furthermore, in many cases it is impractical to obtain a reasonable amount of the synthetic product for use.

The present invention relates to multiple peptide conjugates which comprise multiple peptides, such as antigens, epitopes or other peptides, coupled to a core template. As used herein the term "peptide" refers to compounds comprising two or more amino acids linked by peptide bonds. In some embodiments, the peptides may be from about 2 to about 100 amino acids in length. In other embodiments, the peptides may be from about 10 to about 100 amino acids in length. However, it will be appreciated that the peptides may have any length consistent with their intended use.

The core template preferably comprises a branched compound. Preferably, the core template has at least two branches. However, the core template may have 3, 4 or more than four branches. In fact, the core template may have any number of branches consistent with its intended function.

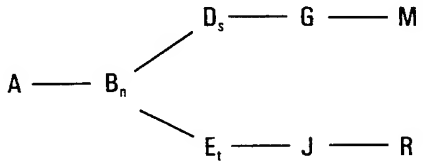
Each branch of the core template contains a first reactive group and a second reactive group, each of which permit peptides to be selectively attached thereto or synthesized thereon. Preferably, the adjacent branches of the core template are spaced at a sufficient distance from one another to minimize the steric hindrance between peptides being synthesized on or attached to the reactive groups on the branches of the core template. Preferably, the peptides are synthesized on the core template. Preferably, the adjacent branches of the core template are separated from one another by the distance occupied by at least four carbon atoms. For example, in some of the embodiments described below, the adjacent branches are linked to the α and ε amino groups of a lysine residue such that they are separated from one another by four carbons.

In one embodiment, the core template comprises a peptide. In this embodiment, the core template may be synthesized using any of the conventional methods for synthesizing peptides, including solid phase synthesis or conventional solution phase chemistry. Synthesis may include the use of any and all protecting strategies, such as the use of t-Boc, Fmoc, Bpoc or other suitable urethane derivatives for blockage of the N-terminal amine prior to coupling or attaching subsequent amino acids in a continuous chain or array.

Synthesis may also be performed by the methodology of convergent solid phase synthesis using protected fragments utilizing acid labile linkers or resins. Convergent chemical ligation of unprotected peptides, fragments or amino acids may also be used to prepare the core template. Thus, the synthesis of the core template may be achieved

entirely by direct, indirect or modular means or a combination of these techniques which couple two or more amino acids together to form conventional peptide bonds or other stable bonds enabling a covalent and stable linkage between two amino acids.

In one embodiment of the present invention, the core template comprises a two branched compound of the following structure:



(Structure A)

wherein:

A is absent or an amino acid;

B is an amino acid;

D is absent or an amino acid;

E is absent or an amino acid;

G is an amino acid;

J is an amino acid;

M is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

R is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

n is 1-10;

s is 0 when D is absent or is 1-20 when D is present; and

t is 0 when E is absent or is 1-20 when E is present.

Preferably, the amino acids in Structure A (i.e. A (if present), B, D (if present), E(if present), G, J, M, and R) are linked to one another via peptide bonds.

Two branched compounds may be synthesized as follows. In a preferred version of the compounds with two branches, the starting material for synthesizing the core template is an amino acid linked to a solid support such as a resin. Thus, in this embodiment, residue A is preferably an amino acid which can be linked to a solid support such as a resin. In a preferred embodiment, residue A of structure A is serine. Preferably, the side chain of the serine used as the starting material for synthesis is tritylated to block its reactivity. The use of serine(Trt) allows modification on the

solid phase after deprotection of the hydroxyl group to produce a highly reactive group for chemical ligation. For example, the hydroxyl group may be oxidized to an aldehyde group or otherwise modified to provide a group suitable for coupling other chemical moieties at this specific site. The C-terminal serine modification can be performed in the solid phase state or as a free peptide.

It will be appreciated that the C -terminal residue may be any residue consistent with its intended use. For example, the serine residue placed at C-terminal end of the core template may be substituted by any number of amino or non amino acids. In the case of Fmoc synthesis for example, the C-terminal serine may be replaced with Lysine(Mtt), Lys(Tfa), Lys(Dde), Cys(mmt), Cys(Acm) as well as many other suitable amino acids or compounds capable of side chain modification. If desired, Fmoc synthesis may be replaced by the conventional t-Boc synthesis. In this case, the C-terminal amino acid may be selected from t-Boc amino acids, such as Cys(Acm), Lys(Dde), Lys(Fmoc), Cys(tbu) or other t-Boc amino acids, with side chain protection corresponding to those listed for Fmoc.

After preparation of the starting material for synthesizing the core template, further peptide synthesis is performed to extend the starting material. For example, if the starting material is Ser(Trt) linked to a resin, additional amino acids may be added thereto to synthesize the core template.

In one embodiment, an initial lysine residue is linked to the Ser(Trt). Thus, residue B can be lysine. This initial lysine forms the branch point. As indicated in Structure A, there may be more than one residue between residue A and the branch point (i.e. n is more than 1). In some embodiments, n is from 1-10. In other embodiments, n is from 1-5. In a preferred embodiment, n is 1.

Thereafter, additional residues are added to residue B of structure A. For example, in some embodiments, residues D and E may be added to extend the chain lengths of the branches. Thus, s and t may be from 1-20. In some embodiments s and t are from 10. In other embodiments, s and t are from 1-5.In one embodiment, residues D and E of structure A are absent (i.e. s and t are 0).

Residues G and J of structure A may be any amino acid. In a preferred embodiment, G and J are lysines. In this embodiment, if residue B is lysine, lysine residues G and J are attached to the α and ε NH_2 groups of residue B to form a branched structure. In one version of the embodiment in which residues G, and J are lysines, the ε NH_2 group of these lysines are blocked with a tert-butoxycarbonyl (t-Boc) group and residues M and R are joined to the α NH_2 groups of these lysines. Alternatively, the α NH_2 groups of lysines G and J may be blocked and M and R may be linked to the ε NH_2 of lysines G and J.

It will be appreciated that the lysines may be substituted with any residues consistent with the intended use. For example, rather than being lysine, residues B, G and J may be derivatives such as Fmoc-Orn(Mtt)-OH, Fmoc-Lys(ivDde)-OH. Residues B, G, and J may also be other diamino compounds having appropriate steric and chemical properties. Preferably, the residues B, G, and J bear a reactive side chain. Preferably, the reactive side chain is spatially separated by at least about four carbon atoms from the chiral center of the molecule. In addition, residues B, G and J are preferably compatible with standard chemical methodologies, such as solid phase and solution chemistry,

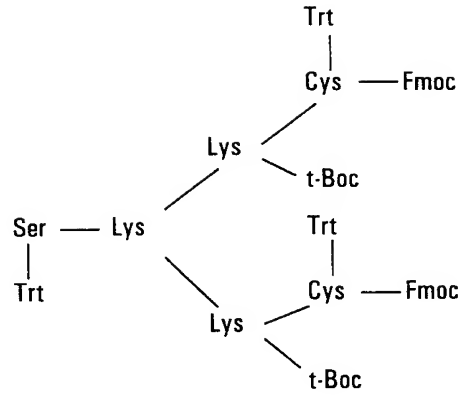
used in the preparation of synthetic peptides. In addition, residues B, G, and J are preferably suitable for use in an orthogonal or non orthogonal synthesis approach using either Fmoc or t-Boc chemistry or using alternate protecting groups for those strategies using both acid and base chemistries. In some embodiments, the residues B, G, and J are spatially arranged such that the level of steric hindrance does not exceed that in the compounds in which residues B, G and J are lysines.

Residues M and R have at least two reactive groups thereon. Each of the reactive groups permits a peptide to be specifically synthesized thereon or attached thereto after synthesis. The reactive groups are designed such that a peptide can be synthesized thereon or a fully synthesized peptide can be attached thereto under conditions in which peptides cannot be synthesized on or attached to the remaining reactive group or groups. For example, the reactive groups may be amino acids modified with appropriate blocking groups such that a single reactive group may be unblocked at a time. In one particular embodiment, one of the reactive groups is deprotected upon acid treatment, while the other reactive group is deprotected upon treatment with base. Thus, the peptide will be synthesized on or attached to only one reactive group at a time.

In a preferred embodiment, residues M and R are cysteines and the first and second reactive groups comprise the thiol group of the cysteines and the amino groups of the cysteines respectively. Preferably, peptides are synthesized on the amino groups of the cysteines and the cysteine side chain thiol is used to form a stable thioether bond with an incoming haloacetyl peptide. In addition, peptides may be synthesized on the amino groups of the cysteines with a reduced risk of epimerization, and, if the thiol groups of the cysteines are blocked during synthesis of the peptides on the amino groups of the cysteines, there is a reduced risk of dimerization during synthesis of the peptides on the amino groups of the cysteines. In one embodiment, the thiol groups of the cysteines are blocked with Trt groups, while the amino groups are blocked with Fmoc.

It will be appreciated that M and R may be amino acids or non-amino acids other than cysteine which provide first and second reactive groups which can be selectively attached to an amino acid or peptide. For example, residues M and R may be amino acids having reactive side chains, such as lysine, serine, threonine, asparagine, aspartic acid, glutamine, and glutamic acid. Preferably, M and R are capable of forming a chemical bond with both the preceding and successive amino acid in the peptide chain. Thus, the covalent bonds between the peptide antigens and base peptide need not be the traditional peptide bond (NHCO) formed between the amino group (NH_2) of one amino acid and the carboxyl (COOH) of another but may be any number of other bonds that insures rigidity, and strength for use in this kind of chemical synthesis. Such couplings may include coupling via thioether formation. Other couplings may also include that of the oxime, thiazolidine, hydrazone, thioester, or other suitable bond formation compatible with all chemistries and groups used in the preparation of the multiple peptide conjugate. M and R preferably have stability and lability characteristics compatible with solid phase or solution syntheses and side chain reactivity capable of protection and deprotection in such manner that the stability and lability of the peptide chains would not be affected.

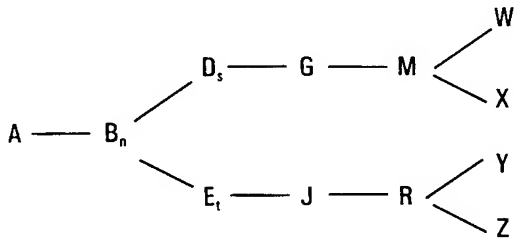
Thus, in one embodiment of the present invention, the core template has the structure



(Structure B)

Structure B has the chemical structure depicted in Figure 2.

In other embodiments, the core template may have more than two branches. For example, the core template may have 3, 4, or more than four branches. For example, the core template may have the structure:



(Structure C)

wherein:

A is absent or an amino acid;

B is an amino acid;

D is absent or an amino acid;

E is absent or an amino acid;

G is an amino acid;

J is an amino acid;

M is an amino acid having two reactive groups thereon which can be attached to an amino acid;

R is an amino acid having two reactive groups thereon which can be attached to an amino acid;

W is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

X is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

Y is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

Z is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

n is 1-10;

s is 0 when D is absent or is 1-20 when D is present; and

t is 0 when E is absent or is 1-20 when E is present.

A, B, D, and E may have any of the identities discussed above with respect to Structure A. The values of n, s, and t may be any of those discussed above with respect to Structure A.

Preferably, the amino acids in Structure C (i.e. A (if present), B, D (if present) , E(if present), G, J, M, R, W, X, Y and Z) are linked to one another via peptide bonds.

G and J may be any amino acid. In some embodiments, G and J are lysines.

M and R may be any amino acids having two reactive groups which can be linked to an amino acid, such that residues M and R form branch points. In preferred embodiments, M and R are lysines. However, M and R may have any of the identities discussed above for residues G and J of Structure A.

W, X, Y, and Z may have any of the identities discussed above for residues M and R of Structure A. In a preferred embodiment, W, X, Y, and Z are cysteines.

It will be appreciated that additional branches may be added by adding additional amino acids having two reactive groups which can be linked to an amino acid at the penultimate positions of the branches. In preferred embodiments, the penultimate amino acids are lysines.

As discussed above with respect to Structure A, the α or ε amino groups of the lysines are then linked to amino acids having a first reactive group and a second reactive group thereon wherein the first and second reactive groups are capable of being selectively attached to an amino acid or peptide. Preferably, the amino acids having a first reactive group and a second reactive group thereon wherein the first and second reactive groups are capable of being selectively attached to an amino acid or peptide are cysteines.

Although the core template structures provided above are amino acids linked by peptide bonds, it will be appreciated that the core template may be any compound, including compounds which are not based on peptide bonds between amino acids, which allows peptides to be selectively synthesized on or attached directly to a single type of reactive group at a time as discussed in more detail below.

Following preparation of the core template, peptides are synthesized on the first reactive groups or fully synthesized peptides are attached to the first reactive groups to generate a base peptide. Preferably, the peptides are synthesized on the first reactive groups. In one embodiment, in which peptides are synthesized on the first reactive groups, a first amino acid residue may be attached to the first reactive groups using any of the synthetic methods familiar to those skilled in the art. The peptide chain may then be extended one residue at a time to generate the

desired peptide using any of the synthetic methods familiar to those skilled in the art. If the first reactive group is blocked, the blocking group is removed prior to linking the first amino acid thereto.

Alternatively, rather than synthesizing the desired peptide one residue at a time starting from the reactive groups on the core template, the desired peptide sequence may be fully synthesized using all conventional methods. Subsequently, the complete peptide is attached to the reactive group on the core template.

Preferably, to reduce steric effects one or more amino acids in the peptides being synthesized on or attached to the first reactive groups are modified with agents which reduce steric effects. Thus, one or more of the amino acids added to the peptide chain during synthesis or included in a fully synthesized peptide may be protected using any of the blocking groups familiar to those skilled in the art. In a preferred embodiment, one or more amino acids linked to the carboxy group of asparagine or aspartic acid residues are incorporated as Hmb protected derivatives in the peptides being synthesized on or attached to the first reactive groups.

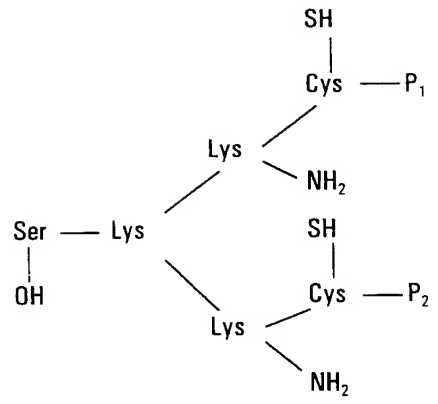
In addition, in some embodiments of the present invention, any cysteine in the peptides being synthesized on or attached to the first reactive groups may have their thiol groups modified with a blocking group. In a preferred embodiment, the thiols are blocked with trityl groups.

In another embodiment, in which the peptides are synthesized on the first reactive groups, the synthesis is conducted in a solvent containing DMSO. The DMSO may be present at a concentration from about 1% to about 20% . In a preferred embodiment, the DMSO is present at a concentration from about 5% to about 12%. In a highly preferred embodiment the DMSO is present at a concentration of about 10%. Alternatively other chaotropic salts, such as KSCN, sodium perchlorate, THT (tetrahydrofuran), 1,4, dioxane, TFE (trifluoroethanol) or LiCl may be used to increase solvation.

In one embodiment, the solvent is the N-methylpyrollidone coupling solvent conventionally used to synthesize peptides. In another embodiment, the solvent may be DMF (dimethylformamide). It will be appreciated that any suitable solvent, including aprotic solvents, may be used.

The synthesis of peptides on the first reactive groups or the attachment of fully synthesized peptides to the first reactive groups on the core templates generates the base peptide. Where the core template has Structure A, the base peptide has peptides attached to the first reactive groups of residues M and R. Where the core template has Structure C, the base peptide has peptides attached to the first reactive groups of residues W, X, Y, and Z.

In the embodiment in which the core template has Structure B, the peptides are selectively synthesized on or joined to the amino groups of the cysteines following deprotection thereof to form the base peptide. For example, in the embodiment in which the core template has Structure B, the base peptide has the following structure after removal of the blocking groups on the thiols of the cysteines:



(Structure D)

wherein P_1 and P_2 are peptides which have been linked to the amino groups of the cysteines. The chemical structure of Structure D is depicted in Figure 3.

If desired, the core template may be cleaved from the solid support following synthesis or attachment of the peptides to the core template. In embodiments in which the thiol group of cysteine is one of the reactive groups on the core template, preferably the cysteine thiol groups on the core template are blocked with an appropriate blocking group such that only these groups are deprotected during the resin cleavage of the peptide by acidolysis. Use of an acid labile protecting group on the cysteine residues in the core template and non acid labile group(s) on all cysteine residues in the peptides which are synthesized on or attached to the core template during synthesis of the base peptide provides compatibility with the sulfhydryl reducing agents used in the final assembly of the multiple peptide conjugate.

However, it will be appreciated that the above measures need not be employed when the peptides attached to the core template during synthesis of the base peptide do not include cysteine residues. In such instances, the thiol blocking group (trityl) on the core template may be replaced with any blocking group compatible with its intended use. For example, Cys(Acm), Cys(tbu), Cys(Mmt), Cys(Npys) or other protected residues could be used in this strategy and appropriate chemical methodologies would be used to deprotect the thiol group when desired.

The base peptide(s) may also be prepared by a chemical or solution synthesis process. In either case, a free peptide can be generated after an acidolysis or other cleavage procedure is performed on resin bound peptide or protected peptide in solution. An alternate approach may also include the synthesis, whether by solid phase or solution chemistry, of protected fragment(s) of either the base or core molecule. These components or fragment(s) produced as free peptides or protected fragments can then be used in convergent synthesis by activation and coupling of two ends of the molecule together followed by suitable methods for isolation. Unlike the protected fragments the coupling of free peptides to the core molecule or other peptides generally will require chemoselective specificity to ensure reactivity at a specific site or residue.

In the embodiment where the core template has Structure B, steric effects are lessened by coupling only to the amino groups of the cysteines on the alpha amino group of opposite lysine positions and maintaining protection on

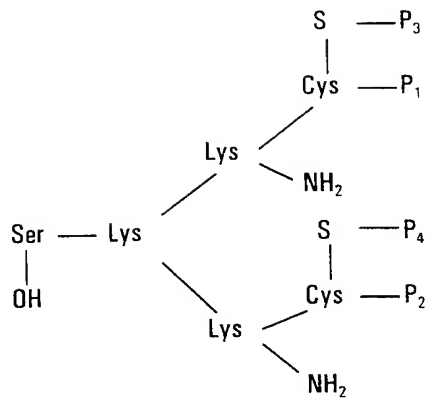
the epsilon position of each respective lysine residue on the tetramer core. Synthesis of two identical peptide chains on the amino groups of the cysteines occurs using this strategy. It will be appreciated that the same methodology may be used to attach four peptide chains to an octameric core template such that the epsilon position of the lysines is protected while the peptides are coupled to cysteines on alternate alpha positions on the lysines in the octameric core template.

Alternatively, peptide chain elongation may be performed from cysteines on the epsilon position of the lysines with the alpha position of the lysines in a blocked state. The results would be identical to that obtained when the cysteines are linked to the alpha positions of the lysines, since the spatial construction of the core template would not be changed in any way.

It will be appreciated that in some embodiments, the amino groups of the two cysteines may be protected with different blocking groups such that different peptides may be synthesized thereon or attached thereto. In such embodiments, the different peptides are sequentially attached to the first reactive groups following deprotection with the appropriate reagents.

Following the synthesis or attachment of peptides on the first reactive groups to generate the base peptide, peptides are selectively synthesized or attached on the second reactive groups to generate a multiple peptide conjugate. Preferably, pre-synthesized peptides are attached to the second reactive groups. The peptides synthesized or attached on the second reactive groups may be the same as or different from those synthesized or attached on the first reactive groups. Thus, in the embodiment in which the core template has Structure A, the multiple peptide conjugate has peptides attached to the first and second reactive groups of residues M and R. In a preferred embodiment, pre-synthesized peptides are attached to the second reactive groups on the base peptide. For example, a haloacetyl group at the end of a pre-synthesized peptide may be attached to thiol groups on cysteine residues in the base peptide.

For example, in the embodiment in which the base peptide has Structure D, the peptides are selectively synthesized or attached on the thiol groups of the cysteines. The peptides synthesized or attached on the thiol groups of the cysteines may be the same as or different from the peptides synthesized or attached on the amino groups of the cysteines. In the embodiment in which the base peptide has structure D, the multiple peptide conjugate has the following structure:



(Structure E)

wherein P_1, P_2, P_3 and P_4 are peptides.

The chemical structure of one embodiment Structure E is shown in Figure 4. In this embodiment, the peptides P_3 and P_4 are linked to the cysteine thiol groups via a haloacetyl group. However, other groups may also be used, or the peptides may be attached directly to the thiol groups in the cysteines.

In some embodiments of the present invention, reactive groups in the peptides which are to be attached to the base peptide to generate the multiple peptide conjugate are protected with a blocking group which prevents them from being reactive under the conditions in which they are attached to the base peptide. In other words, the blocking groups in the peptides which are to be attached to the base peptide to generate the multiple peptide conjugate are different than the blocking groups on the reactive groups in the base peptide such that the reactive groups in the base peptide can be selectively deprotected.

For example, in the embodiment in which the base peptide has Structure D in which the thiol groups of the cysteines are the reactive groups to which the peptides are to be attached during synthesis of the multiple peptide conjugates, any cysteines present in the peptides which were attached to the core template during synthesis of the base peptide may be cysteine (tbuthio). The use of Cys(tbuthio) insures site directed coupling by the haloacetyl peptide to the thiols. It will be appreciated that the internal Cys(tbuthio) may be replaced with other SH protection compatible with the chemistry employed to synthesize the multiple peptide conjugate.

It will be appreciated that one or more of the peptides in the multiple peptide conjugates may also include one or more peptides adjacent to the peptides P1, P2, P3, and P4. For example, the peptides may comprise one, two, three, four, five or more than five peptides adjacent thereto. Alternatively, the peptides may comprise about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 70, or more than 70 peptides adjacent to the peptides P1, P2, P3, and P4.

In some embodiments of the present invention, the peptides included in the multiple peptide conjugate comprise agents, including antigens or epitopes, which are capable of eliciting an immune response in an individual. In some embodiments of the present invention, the peptides comprise antigens from a pathogenic organism. It will be

appreciated that in some embodiments the peptide antigens from pathogenic organisms may have amino acids or non-amino acids inserted therein which function as linkers between two antigenic peptides which are fused together or which provide any desirable attribute to the antigenic peptide.

In particular embodiments of the present invention, the peptides attached to the core template are from the causative agents of malaria and/or AIDS. For example, the peptide attached to the core template may be from the HIV-1 Tat protein. Preferably, the peptide attached to the core template comprises an antigenic epitope from the HIV-1 Tat protein.

In some embodiments, one or more of the peptides in the multiple peptide conjugate comprises a portion of the Tat protein which is responsible for mediating one or more activities of the Tat protein, such as replication of HIV-1, transcription from the HIV-1 LTR, induction of cytokine production, increased activity of the F transcription factor, or increased angiogenic activity in assays such as the chick CAM assay (Gho and Chae. *J. Biol. Chem.* 272: 24294 (1997)). Preferably, the portion of the HIV-1 Tat protein also includes one or more antigenic sites therein.

Fragments of the HIV-1 Tat protein which are responsible for mediating one or more activities of the Tat protein are described above. In some embodiments of the present invention, one or more of the peptides in the multiple peptide conjugate constitute SEQ ID NOS:1-21. In other embodiments, one or more of the peptides in the multiple peptide conjugates may comprise two, three, four, five, or more than five peptides containing Tat functional domains linked to one another. In some embodiments, the two, three, four, five, or more than five peptides are spaced from one another by a linker comprising one or more amino acids or non-amino acids.

In another embodiment, one or more of the peptides in the multiple peptide conjugate comprises a peptide from *Plasmodium falciparium*, the causative agent of malaria. Preferably, the peptide from *Plasmodium falciparium* has at least one antigenic site therein.

In one embodiment, one or more of the peptides in the multiple peptide conjugate comprises a T3 peptide from *Plasmodium falciparium*. For example, the peptide may comprise the sequence NENLDDLDEGIEKSSEELSEEKI (SEQ ID NO: 81). In some embodiments, one or more of the residues which are linked to the carboxyl groups of asparagine or aspartic acid residues in the T3 peptide may be Hmb derivatives as indicated by underlining in Figure 5A.

In another embodiment, one or more of the peptides in the multiple peptide conjugate comprises a T1 peptide from *Plasmodium falciparium*. For example, the peptide may comprise the sequence LTMSNVKNVQTNFKSLLRNLGVS (SEQ ID NO: 82). In some embodiments, one or more of the residues which are linked to the carboxyl groups of asparagine or aspartic acid residues in the T1 peptide may be Hmb derivatives as indicated by underlining in Figure 5A.

In still another embodiment, one or more of the peptides in the multiple peptide conjugate comprises an MSP-1 peptide from *Plasmodium falciparium*. For example, the peptide may comprise the sequence VTHESYQELVKKLEALEDAV (SEQ ID NO: 83). In some embodiments, one or more of the residues which are linked to the carboxyl groups of asparagine or aspartic acid residues in the MSP-1 peptide may be Hmb derivatives as indicated by underlining in Figure 5A.

In yet another embodiment, one or more of the peptides in the multiple peptide conjugate may comprise the CSP peptide from *Plasmodium falciparium* (Nardin et al., *Vaccine* 16:590-600 (1998)). For example, the peptide may comprise the sequence NANPNANP (SEQ ID NO: 84). In some embodiments, one or more of the residues which are linked to the carboxyl groups of asparagine or aspartic acid residues in the CSP peptide may be Hmb derivatives as indicated by underlining in Figure 5A.

As discussed above, one or more of the peptides in the multiple peptide conjugates may comprise an immunogenic peptide from any isolate or strain of *Plasmodium falciparium*.

In some embodiments of the present invention, one or more of the peptides in the multiple peptide conjugate comprises a peptide which has one, two, three, four, five or more than five amino acid substitutions relative to a reference T3, T1, MSP-1 or CSP peptide. The substituted peptides may be produced using conventional techniques of peptide synthesis.

In yet another embodiment, one or more of the peptides in the multiple peptide conjugate comprises a fusion between two immunogenic peptides from *Plasmodium falciparium*. In some embodiments, the two peptides are spaced from one another by a linker comprising one or more amino acids or non-amino acids. For example, one or more of the peptides in the multiple peptide conjugate may cormprise a fusion peptide comprising the CSP and T3 peptides such as a peptide comprising the sequence NANPNANPNENLDDLDEGIEKSSEELSEEKI (SEQ ID NO: 85). In some embodiments, one or more of the residues which are linked to the carboxyl groups of asparagine or aspartic acid residues in the fusion peptide may be Hmb derivatives as indicated by underlining in Figure 5A.

In yet another embodiment, one or more of the peptides in the multiple peptide conjugate comprises a fusion between more than two immunogenic peptides from *Plasmodium falciparium*. In some embodiments, one or more residues linked to the carboxy groups of asparagine or aspartic acid residues may be Hmb derivatives.

It will be appreciated that the multiple peptide conjugates may include any peptide from HIV-1, *Plasmodium falciparium*, or any other organism, which is capable of inducing an immune response. Thus, the multiple peptide conjugates are not limited to the peptides specifically enumerated above.

In some embodiments, the multiple peptide conjugates synthesized as described above may be used to induce an immune response in an individual. The multiple peptide conjugates may be administered via any of the routes traditionally used to administer compositions which induce an immune response, including intraperitoneal administration, intramuscular administration, intradermal administration, intravascular administration or nasal administration.

If desired, the multiple peptide conjugates may be administered along with an adjuvant to enhance the level of the immune response. A variety of adjuvants suitable for use in compositions for inducing an immune response are familiar to those skilled in the art, including aluminums like hydroxide and phosphate.

The multiple peptide conjugate may be administered in a physiologically acceptable carrier. A variety of carriers suitable for use in compositions for inducing an immune response are familiar to those skilled in the art. Such carriers are nontoxic to recipients at the dosages and concentrations employed. For example, the carriers may contain

saline, buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrins or chelating agents such as EDTA, glutathione or other stabilizers and excipients.

The multiple peptide conjugates are administered at a dosage and frequency sufficient to induce a desired level of immune response. Preferably, the level of immune response is sufficient to protect an uninfected individual from infection or to reduce or eliminate the symptoms of infection in an infected individual. It will be appreciated that the dosage and frequency of administration of the multiple peptide conjugates will vary depending on the age, weight, and condition of the individual. For example, the dosage sufficient to induce a desired immune response may range from about 1 μg-100 mg or more, more preferably from about 25 μg-100mg or more. However, other dosages may also be used.

In some embodiments, the multiple peptide conjugates may comprise several antigens from a pathogenic organism. For example, two or more different antigenic peptides may be attached to the core template.

Figure 6 illustrates one embodiment of the synthetic process which was used to prepare a multiple peptide conjugate. In the embodiment illustrated in Figure 6, solid phase peptide synthesis followed by thiol coupling chemistry was utilized to synthesize and construct the MPC molecules. In the embodiment of Figure 6, a core template that serves as primary anchor for the synthesis of a base peptide and subsequent attachment of additional peptides through specific site directed coupling was synthesized using solid phase methods. For example, the core template may have the structure illustrated in Figure 7A.

Initially, Serine(Trt) representing the C-terminal of the MPC was attached to the solid support followed by the sequential addition of multiple branched lysine residues forming a tetrameric core whose epsilon amino groups remain blocked throughout the synthesis process. Cysteine, whose side chain thiol group serves to form a stable thioether in the final assembly of the MPC, was then coupled to the dual branched lysines forming a completed core template.

Example 6 describes the synthesis of the synthesis core template having the structure shown in Figure 7A.

Example 6

The core template was synthesized on an ABI Model 430 peptide synthesizer using Fmoc chemistry(Merrifield, In *Peptides: Synthesis, Structure and Applications* (Gutte, Ed.), Academic press, San Diego, pp93 (1995); Atherton and Sheppard, *Solid-phase peptide synthesis*, IRL Press, Oxford, (1989)) mediated by HBTU/HOBt activation on the Rink Amide [4,2',4'Dimethoxyphenyl-Fmoc-aminomethyl] phenoxyacetamido-norleucyl-MBHA resin, 0.45 mmol/g (Novabiochem, La Jolla, CA) at the 0.25 mmol scale for synthesis. Bulk amino acids derivatives were

purchased from Novabiochem and 1.0 mmol of each desired residue was used initially in the coupling reaction. Double couple or recoupling required two or more millimole per residue. For the core template, amino acids were incorporated with the following side chain protection: Lys(Fmoc), Lys(t-Boc), Ser(Trt), and Cys(Trt). Side chain protection used in all other peptides were: Glu(Otbu), His(Trt), Asp(OtBu), Cys(Trt), Lys(t-Boc), Asn(Trt), Arg(Pmc), Ser(tBu), Cys(tButhio), Lys(Fmoc), Val(Fmoc Hmb), Leu(Fmoc Hmb), Gln(Trt), Trp(t-Boc).

As illustrated in Figure 6, the second step of assembly involves the synthesis of a base peptide consisting of one or more antigens. In one embodiment, the base peptide may be generated by synthesizing the antigens on the core template. However, as discussed above, in other embodiments, the complete antigenic peptides may be synthesized first and then attached to the core template.

For example, the base peptide may have the structure shown in Figure 7B. The base peptide constructs were synthesized by synthesizing the antigenic peptides listed in Figure 5A and 5B on the core template as described in Example 7.

Example 7

The base peptides consisting of antigens from malaria and HIV-Tat protein were synthesized on the core template following characterization of the core template. These base peptides were subsequently used to prepare four MPCs. Hmb derivatives were incorporated at specific residues as indicated by underlining in Figure 5A in order to reduce potential aggregation in the growing peptide chains (Johnson et al., *J. Chem. Soc. Chem. Commun.* 4:369-372(1993); Johnson et al., *Tetrahedron Letters* 35:463-466 (1994); Packman et al., *Peptide Research* 7: 125-131(1994); Quibell et al., *J. Am. Chem. Soc.* 117:11656-11668(1995); Quibell et al., *J. Chem.Soc., Perkin Trans.* 1:1227-1234 (1996)). The coupling time for a single coupling was 1 hour. Coupling efficiencies were monitored using the Kaiser or TNBS test after completion of each coupling step to insure greater than 99% completion. Double couple/recouple cycles were introduced when necessary. Cycles which failed to achieve at least 99% after a double/recouple cycle were capped with 2mmol benzoic anhydride to terminate any unreacted amines. An antigen from *Plasmodium falciparum* (LSA-1) (Connelly et al., *Infection and Immunity* 65:5082-5087(1997)) previously identified as T3 was used to construct the base peptide T3. The T3-CSP base peptide was synthesized using T3 base peptide as its template following removal of 1/2 of the T3 peptidyl resin from the synthesizer reaction flask after deprotection thus allowing the synthesis to be continued with an additional eight residues added per branch of the T3 construct. Both T3 and T3CSP base peptides were subjected to cleavage by acidolysis and purification. The HIV-Tat base peptide

Tat-2-Tat-3 was obtained by direct solid phase synthesis under similar conditions as described at the 0.25 mmol scale followed by cleavage and purification.

Peptides were then deprotected and cleaved from the resin as described in Example 8.

Example 8

Following deprotection of the N-terminal amino group, the base peptides were cleaved with a cocktail of TFA, water, EDT, TIS, phenol, thioanisole (88:4:2:1:1:4). Following cleavage, base peptides were precipitated and washed in cold Tert-butyl Methyl Ether. Prior to further processing, peptides were dissolved in water or 0.1% TFA or acetic acid followed by the addition of 1.0 ml -mercaptoethanol.

The crude peptides were purified by RP-HPLC as described in Example 9.

Example 9

Base peptides were purified by RP-HPLC using 6.8 x 25 cm Vydac C4, C8, or C18 columns (The Separation Group, Hesperia, CA) with a gradient of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. Following HPLC purification, the acetonitrile was removed by rotary evaporator and the peptides were lyopholized and stored at -20° or -70°C (haloacetyl peptides) until needed.

Fractions containing the desired peptide as determined by mass spectral analysis, were pooled and lyophilized. Identities of the base peptides were established by MALDI-MS. Mass spectrometry was used to identify and/or confirm the molecular mass of the various peptide species. Mass analysis was performed using a Voyager DE-RP MALDI-TOF mass spectrometer (Perseptive Biosystems, San Jose, CA). The ionization matrix for the analysis was carried out in 10 mg Sinapinic acid/ -CHCA dissolved in 50% acetonitrile/0.1% TFA. All mass analyses were performed in the positive ion mode. The observed molecular ion for each construct was found to be consistent with that obtained from the theoretical sequence (Table A).

Table A

Mass spectral analysis of base peptides and multiple peptide conjugates

MPC	Mass [MH+] Theoretical	Observed
T3 (base peptide)	5,930.56	5,931
T3-CSP (base peptide)	7,515.15	7, 513
Tat-2-Tat-3 (base peptide)	7,303.65	7, 306
T1-T3	11,139.24	11, 144
T1-CSP-T3	12,723.73	12, 737
T3-MSP	10,615.03	10, 711
Tat-1-Tat-2-Tat-3	11,997.35	12,460

*Theoretical mass reported for the tetrameric MPC molecules.

The amino acid sequence and the relative degree of purity of the base peptides were established by Edman sequence analysis (Liu et al., *Anal Biochem.* 182:383-387 (1989)) on an Applied Biosystems Model 494 sequencer, Foster City, CA as well as quantitative amino acid compositional analyses to determine specific molar ratio for each residue. The molar ratio of each amino acid was found to be consistent with that of the theoretical values as indicated in Table B.

Table B

Amino acid composition of the base peptides

Amino Acid	*Theoretical		Experimental	
	T3-CSP	Tat-2-Tat-3	T3-CSP	Tat-2-Tat-3
Asx	9	1	8.7	0.8
Thr	-	1		0.7
Ser	4	4	3.6	3.5
Glx	7	6	6.8	5.7
Pro	2	4	2.0	3.6
Gly	1	1	0.8	1.2
Ala	2	2	2.0	2.0
Cys**	2	2	1.8	1.8
Val	-	-		
Met	-	-		
Ile	2	1	1.5	0.8
Leu	3	-	2.7	
Tyr	-	-		
Phe	-	-		
His	-	4		3.7
Lys	5	3	4.7	2.8
Trp	-	1		0.8
Arg	-	4		3.8

*Theoretical = peptide plus Core molecule

**Cysteine determined as cysteic acid

Linear haloacetyl peptides for attachment to the base peptides were synthesized as described in Examples 10 and 11.

Example 10

Peptides comprising malarial antigens were synthesized as described above using standard ABI Fmoc cycles with a final cycle added for modification of the N-terminal amino group with a haloacetyl group (Robey and Fields, *Anal Biochem.* 177:373-377 (1989)). Briefly, 1.0 mmol N-bromoacetyl-NHS ester, Cl_2Ac_2O or Br_2Ac_2O which is prepared by dissolving 1.0 mmol $BrCH_2COOH$ in DCM to which 0.5mmol DCC in DCM is added with mixing. The reaction was carried out for 30 minutes at room temperature and the solution is filtered to remove the DCU precipitate formed. The activated amino acid residue [active ester/anhydride] was then added to the reaction vessel containing the resin bound deprotected peptide and allowed to couple for 1 hour at room temperature. Coupling of the anhydride was carried out in DCM. Following synthesis, peptides were cleaved from the resin according to a specific cleavage protocol described (Boykins et al. *Cytokine* 11:8-15 (1999)) followed by purification by RP-HPLC. The peptides were lyophilized and stored at -70°C until needed.

Example 11

The HIV-Tat peptides (Boykins et al, *J. Immunol.* 163:15-20 (1999)) were synthesized essentially the same way as the malaria peptides, except that all cysteine sulfhydryl residues of Tat-1 peptide were protected with the tButhio blocking group (Figure 5B). Chloroacetylation of the amino terminal end of the peptide was chosen specifically for the Tat peptide to minimize reactivity with other highly reactive side chains present in both the base and linear peptides during final assembly of the Tat MPC. Cleavage of this molecule produced a stable N-chloroacetyl peptide whose side chain thiol groups remained blocked throughout the purification. Subsequently, the haloacetyl linear peptide Tat-1 was coupled to the base peptide Tat-2-Tat-3. The sulfhydryl protection was released following coupling of the haloacetyl peptide to the previously deprotected cysteine residues on the base peptide. Deprotection was accomplished with an excess of Bu_3P reagent as used earlier in the assembly process.

The linear haloacetyl peptides were then cleaved as described in Example 12.

Example 12

Haloacetyl peptides were cleaved in a cleavage cocktail consisted of TFA, water, phenol (90:5:5). Thiol scavengers were not used since thiol compounds could react with the haloacetyl moiety as well as could result in the deprotection of the Cys (tButhio) in the Tat linear peptide.

The haloacetyl peptides were purified and characterized as described in Example 9 above.

In the final phase of the assembly, with identities established by mass spectral and amino acid analyses, HPLC purified linear or S-protected haloacetyl peptides comprising one or more functional epitopes were then coupled to the cysteine thiol groups on the base peptide to generate an MPC molecule having the structure shown in Figure 7C. Examples 13 and 14 describe the coupling of the malarial or HIV Tat peptides to the base peptide.

Example 13

The base construct and the linear haloacetyl malarial peptide were coupled as follows. Into a 50 ml teflon flask fitted with a nitrogen tube, the base peptide was dissolved in 0.5M $NaHCO_3$ or 0.1-1M Tris/guandine-HCl pH 8.0 buffer as needed. A solution of Bu_3P in 1-propanol or TCEP was freshly prepared. The haloacetyl peptides were

dissolved in 1-2 ml (0-6M) guandinine-HCl and added to the reaction flask under nitrogen. For example, to 10 mg base peptide T3 (1.7 M) 0.6 M reducing agent was added for reduction of the base cysteinyl residues. The solution was placed on a stirrer for 1 hour followed by the addition of 0.8 mg (0.29 M) haloacetyl peptide (T1) to the reaction mixture. The coupling reaction was then carried out for 1.5-3 hours at 25°C for a bromoacetyl peptide and up to 6 hours when using the chloroacetyl derivative. The reaction mixture was monitored with MALDI-MS for the presence of conjugate material. -mercaptoethanol (0.2 ml) was then added to the reaction flask. The conjugate mixture was then desalted by RP-HPLC and further purified by size exclusion chromatography to isolate the desired multiple peptide conjugate.

Example 14

The base peptide and linear (sulfhydryl protected) HIV-1 Tat haloacetyl peptide were coupled as follows. HIV-Tat peptide (Tat-1) peptide contains seven protected cysteine residues. A similar strategy, as previously described, was used to couple the N-chloroacetyl modified HIV-Tat peptide to the base peptide Tat-2-Tat-3. The base peptide was dissolved in 1M Tris/6M guanidine HCl buffer, pH 8.0. A fivefold excess (based on thiol content) of Bu_3P in 1-propanol was added to effect reduction of the base cysteine residues. Essentially, to 10 mg (1.4 M) of the base peptide Tat-2-Tat-3, 0.25 M Bu_3P reagent was added. Following reduction of the cysteinyl residues in the base molecule, 1.6 mg (0.5 M) chloroacetyl peptide was dissolved in 6M guanidine-HCl and added to the reaction mixture. The solution was adjusted to 20% (v/v) with 1-propanol to increase solubility of the Tat haloacetyl peptide, and allowed to proceed 2-6 hours under nitrogen. Reaction mix was again monitored qualitatively by MALDI-MS by observing the presence of a signal consistent with the expected mass of the desired MPC. Following conjugation of the base and haloacetyl peptide, excess Bu_3P (1.9 mol) was added for final deprotection of the cysteine residues. The deprotection was carried out for 2 hours at 25°C. The conjugate mixture was then desalted by RP-HPLC as described below.

Example 15

The peptide conjugate mixtures were desalted by RP-HPLC using a C_{18} column (10 μ, 19 mm x 150 mm) Waters, Milford, MA using a one step gradient of 0.1% TFA/H_2O and 10% acetonitrile/H_2O to elute the low molecular weight salts followed by 70% acetonitrile to elute the MPC or any unconjugated peptides. The conjugated mixtures were then lyophilized and stored at -20°C.

The MPC, thus formed was further characterized by mass spectroscopy, size exclusion chromatography, and SDS-gel electrophoresis. Mass spectroscopy was performed as described in Example 9 above. Example 16 describes the size exclusion chromatography analyses, while Example 17 describes the SDS gel electrophoresis.

Example 16

Size exclusion chromatography was performed using a Diol-S5 column (YMC, Wilmington, NC) to isolate the desired MPC peptide from the reaction mixture. The elution buffer consisted of 0.1M phosphate buffer, 0.2 M NaCl, pH 7.0, containing 0.02 M sodium azide. The column was standardized using a GPC standard molecular weight mixture obtained from Bio Rad (Hercules, CA).

Example 17

The MPCs were subjected to SDS-polyacrylamide gel electrophoresis using 10-20% Tricine gels (Novex, San Diego, CA) under reducing conditions. After completion of electrophoresis, gels were stained with Commassie blue to visualize the position of the MPCs.

The yields of the various base constructs and the efficiency of conjugation are summarized in Table C and Table D, respectively. As shown in Table D, the CMC values indicate that the extent of conjugation was nearly complete. Under ideal conditions, 2 mole of CMC/mole of MPC should be released during hydrolysis of the conjugate molecule.

Table C

Summary of yields for the base peptides

	Yield (mg)	
Base peptide	Crude	HPLC Purified
T3	680	258
T3-CSP	845	302
Tat-2-Tat-3	1750	490

*All yields calculated from acid hydrolysis of protein content as determined by amino acid analysis.

Table D

Relative Degree of Conjugation

Haloacetyl peptide +	Base peptide	*mole CMC/mole MPC
T1	T3	1.5
MSP-1	T3	1.8
Tat-1	Tat-2-Tat-3	1.4
T1	T3-CSP	1.5

*Calculations based on determination of carboxymethylcysteine [CMC] after acid hydrolysis of conjugated peptide by amino acid analysis.

Mass anlaysis of the T1-T3 construct produces an ion signal centered at 11,144 m/z. Analysis of the T1-CSP-T3 produces a similar ion cluster centered at 12,737 m/z. The level of purity for each MPC was determined by analytical reverse phase HPLC on a Vydac C18, 5μ column.

Molecular weight of the MPCs was determined by SDS-PAGE under reducing conditions. The apparent molecular weights of malaria peptide conjugates T1-T3, T1-MSP-1, and T1-CSP-T3 were observed to be 10 kDa, 12 kDa, 13 kDa and that of the HIV-1-Tat MPC (Tat-1-Tat-2-Tat-3) to be 12 kDa, respectively. Overall purities of the tetrameric MPC molecules were greater than 80% with the absolute percent dependent upon isolation and removal by size exclusion chromatography (SEC) of the base and linear peptides used as reactants. Analysis of the final MPC molecules by mass spectroscopy and SDS-gel electrophoresis indicated the presence of material corresponding to a trimeric species. Analysis of these data suggested the presence of a trimer could have been the result of incomplete coupling to the thiol groups on the core molecule. In all the MPCs reported here, the trimer represented approximately 20% relative to the tetramer.

As discussed above, aggregation and steric hindrance have resulted in microheterogeneity in multiple peptide conjugates made using other techniques. In order to minimize these possible deleterious effects during chain assembly, a small amount of DMSO was added to the NMP solvent and several Hmb amino acids were incorporated throughout the peptide chain in the syntheses described above. Furthermore, in the methods described above, incorporation of a serine residue as the C-terminal amino acid with its side chain protected by a trityl group allows ease of removal and further modification prior to cleavage from the solid support. The side chain of this residue could be useful in the attachment of one or more antigens, adjuvant or other component.

As discussed above, assembly of a tetrameric core template and coupling to alternate lysine residues with the epilson position remaining blocked may provide greater access to the resin bound amino groups during the acylation and deprotection reactions, thus ensuring a higher degree of coupling efficiency at each cycle especially when coupling residues with bulky side chains. In the above-described syntheses of all base peptides, greater than 99% coupling yields for each residue were obtained as determined by Kaiser or TNBS assay.

It should be noted that while the peptide lengths described above are 31 residues per branch or less on the base peptide, peptide lengths of 50 or more residues per branch may be achieved with similar levels of purity and yield using the above-described methodology.

The above techniques employ nucleophilic substitution of alkyl halides (Lundblad, *Techniques in protein modification*, CRC Press, pp63-96 (1995); Means and Feeney, *Chemical modification of proteins,* Holden-Day, Inc., pp105-138.(1971)). This chemistry has been used extensively in the conjugation of proteins and peptides that result in the formation of stable thioether bonds (Kolodny and Robey, *Anal Biochem* 187: 136-140 (1990)). A faint lower band consistent with the molecular weight of a trimeric species (i.e., the base peptide and one linear peptide coupled) was present in the mixture with the desired tetramer molecule in the case of the malaria MPCs. Mass analysis by MALDI-TOF and ESI-MS also confirmed this finding. In the case of Tat MPC, similar observations were made. However, the average yield of the MPC constructs was greater than 70% tetramer.

The presence of additional species may be the result of limited solubility of the reactants or steric effects thereby limiting accessibility to the thiol group and therefore reducing conjugation. In the above methods, both Tris and bicarbonate buffers were utilized in addition to guandine hydrochloride in order to carry out the thiol coupling step. However, the buffer used to solubilize the base and linear peptides should be selected to optimize the conditions for the thiol reaction.

Measurement of the CMC ratio was performed to determine the degree of conjugation (Kolodny and Robey, *Anal Biochem.* 187: 136-140 (1990)). Slightly less than 2 moles CMC/mole of each MPC construct were present. Under ideal conditions 2 moles of CMC/mole of conjugate should be liberated upon acid hydrolysis. Among the many factors that may inhibit production of the tetramer molecule are solubility of the peptides and steric hindrance. Some of these factors could contribute to variable yields which, in addition, could be dependent upon the physical parameters of the individual species. To facilitate the solubility of the chloroacetyl Tat peptide [Tat-1], increasing amounts of 1-propanol were added. In the case of the more hydrophobic peptides, 1M Tris/6M guandine-HCl minimized the formation of the trimeric MPC. This was particularly true for the MPC containing the Tat-3 peptide, where the overall yield was somewhat lower. The lower yield of the Tat MPC may result from the release of a small number of thiols, Cysteine[tButhio] in the peptide sequence, following addition of the haloacetyl peptide into the reaction mixture containing the base peptide Tat-2-Tat-3. The release could occur as a result of the initial reduction of the base cysteinyl residues with Bu_3P.

Poor solubility of the Tat MPC may be a factor in the SEC analysis to isolate only the MPC and remove the unreacted starting peptides (base and linear peptide). Therefore, the Tat MPC has higher levels of these peptides which may be a factor in suppression of the ionization of the MPC when analyzed by MALDI-MS. However, these low intensity signals observed appear to be consistent with the expected molecular mass indicating the presence of both tetramer and trimer species.

In summary, the above strategies for synthesizing MPCs provide several benefits. The above methods facilitate synthesis of a well defined core template molecule that minimizes steric hindrance by coupling to only two branches per synthetic cycle. While core molecule spacing is similar to that used in the synthesis of a tetramer, only two residues are assembled per cycle at alternate amino groups on the tetrameric core with the opposite positions remaining protected throughout the synthesis of the base molecule.

In addition, in some of the embodiments described above, a serine residue with a trityl group used as side chain protection is incorporated into the core template. The serine residue can be modified on the solid phase for greater functionality without cleavage of the peptide resin link, thereby permitting the attachment of antigens or an adjuvant molecule if desired. Furthermore, as discussed above, synthesis of a base molecule with specific thiol sites for attachment of additional peptides allows site directed coupling of the linear peptides.

In addition, solubility of the peptide chains may be improved by modifying the N-methylpyrollidone coupling solvent by adding 10% DMSO, thereby enhancing the acylation and/or deprotection reaction and improving coupling efficiency. Furthermore, addition of Hmb protected amino acids at specific points in the peptide chain further minimizes aggregation of the growing peptide chains thereby minimizing the risk of lower yields in the desired peptide.

In addition, in some instances the homogeneity of the product may be enhanced by attaching blocking groups to the side chains of one or more amino acids in the haloacetyl peptides to be attached to the base peptide. For example, in the examples above, a tButhio side chain blocking group was used on all cysteinyl residues in the haloacetyl peptides. Internal cysteinyl residues were selectively deprotected only after formation of the final multiple peptide conjugate.

The above methods permit further side chain modification to the lysine and cysteine residues on the core template, thereby permitting alternative methods of selectively attaching peptides to the core template or the core peptide. For example, the reactive groups on the core template can be selected so that one reactive group is deprotected upon treatment with base while the other reactive group is deprotected upon acid treatment. For example, an alternate use of the core template functionality is in the replacement of the t-Boc group at the branched lysine position by a quasi-orthogonally protected lysine derivative such as Fmoc-Lys(Dde) or Fmoc-Lys(ivDde)-OH. The use of such derivatives allows selective deprotection with dilute hydrazine or other similar amines without cleavage from the solid support. The use of Lys(Mtt) or Lys(Tfa) allows selective deprotection with dilute acid solutions. Similarly, the use of Cys(Mmt)-OH as a replacement for the current Cys(Trt) allows the selective deprotection of the thiol with dilute acid, thereby enabling the sulfhydryl to be readily exploited to produce a thioether linkage or other reactions involving the thiol.

Thus, the basic core molecule described above provides increased versatility in the use of multiple strategies for the covalent attachment of additional peptide antigens, adjuvant components, or other desirable functionalities.

The methodology described herein produces well characterized multiple peptide conjugates useful in the development of subunit vaccines in significant yields for important commercial applications.

PART III: HIV-1-TAT-MULTIPLE PEPTIDE CONJUGATE

Background of the Invention

Infection with HIV is responsible for a large number of deaths annually and represents a significant threat to the public health. Despite continuous efforts by a number of laboratories, little progress has been made towards the development of a safe and effective vaccine to control progression of HIV disease. Most strategies have failed due to the huge diversity in predominant immunologic epitopes of gp120 and gp41 of this heterogeneous virus; this diversity occurs between HIV-infected individuals and sometimes even within a single host. (Berman et al., *Nature*, 345:622 (1990); Burton and Moore, *Nature Med.*, 4:495 (1998); Stott et al., *J. Gen. Virol.*, 79:423 (1998)).

Neutralization of extracellular Tat, a regulatory protein of HIV-1, by a therapeutic vaccine approach for the treatment of AIDS has been proposed by a number of investigators. (Cafaro et al., *Nature Med.*, 5:643 (1999); Caselli et al., *J. Immunol.*, 162:5631 (1999); Cohen et al., *Proc. Natl. Acad. Sci. USA*, 96:10842 (1999); Lambert, *J. Hum. Virol.*, 1:249 (1998); Zagury et al., *J. Hum. Virol.*, 1:282 (1998); Gringeri et al., *J. Hum Virol.*, 1:293 (1998); Goldstein, *Nature Med.*, 2:960 (1996)). Tat protein is produced by HIV-infected cells and is believed to be involved in the progression of HIV infection, development of Kaposi's sarcoma, immunosuppression, apoptosis of normal cells, and various neurological disorders. (Ensoli et al., *J. Virol.*, 67:277 (1993); Chang et al., *J. Biomed. Sci.*, 2:189 (1995); Chang et al., *AIDS*, 11:1421 (1997); Chen et al., *J. Biol. Chem.*, 272:22385 (1997)). It has recently been shown that many of the effects of HIV infection of monocytes can be mimicked by treatment of these cells with extracellular HIV-Tat protein. (Dhawan et al., *J. Immunol.*, 154:422 (1995); Lafrenie et al., *J. Immunol.*, 156:1638 (1996); Lafrenie et al., *J. Immunol.*, 157:974 (1996); Lafrenie et al., *J. Immunol.*, 159:4077 (1997); Albini et al., *J. Biol. Chem.*, 273:15895 (1998); Weeks, *Int. J. Molec. Med.*, 1:361 (1998)). Therefore, immunologic obstruction of Tat protein by prophylactic immunization of HIV-infected individuals could reduce viremia and thereby prevent or delay the onset of progression to AIDS. Neutralization of extracellular Tat could also partly restore immune function in AIDS patients. Recent studies have proposed Tat toxoid or oxidized Tat protein as potential candidates for therapeutic AIDS vaccines. (Cafaro et al., *Nature Med.*, 5:643 (1999); Caselli et al., *J. Immunol.*, 162:5631 (1999); Cohen et al., *Proc. Natl. Acad. Sci. USA*, 96:10842 (1999); Lambert, *J. Hum. Virol.*, 1:249 (1998); Zagury et al., *J. Hum. Virol.*, 1:282 (1998); Gringeri et al., *J. Hum Virol.*, 1:293 (1998); Goldstein, *Nature Med.*, 2:960 (1996); Girard et al., *C. R. Acad. Sci. III*, 322:959 (1999); Le Buanec et al., *Biomed. Pharmacother.*, 52:431 (1999)). However, like other viral proteins, there are mutational changes in Tat protein among various viral isolates. The use of full-length recombinant Tat protein from a single HIV-1 strain as a vaccine may, therefore, not be sufficient for effective treatment of individuals infected with different HIV-1 isolates. Moreover, crucial functional epitopes may not be immunodominant. A subunit vaccine targeting conserved, functionally critical epitopes could resolve these problems.

The mechanisms by which HIV-Tat protein promotes viral pathogenesis primarily involve highly conserved cysteine-rich and basic domains in various HIV-1 isolates. (Boykins et al., *J. Immunol.*, 163:15 (1999); Albini et al., *J. Hum. Virol.*, 2:179 (1999); Mitola et al., *J. Virol.*, 74:344 (2000)). Neutralization of these domains by epitope-specific antibodies could provide selective targeting of Tat to reduce progression of HIV infection.

Summary of the Invention

The present invention is directed to a novel highly immunogenic synthetic multiple peptide conjugate constituting functional domains Tat21-40 and Tat53-68 from HIV-1 group M plus Tat9-20 from HIV-1 group O of the HIV-Tat protein (HIV-1-Tat-MPC). Vaccination of mice with HIV-1-Tat-MPC induces an effective immune response to all three functional domains. Anti-HIV-1-Tat-MPC antibodies efficiently inhibit Tat-induced viral activation in monocytes infected with HIV_{Ba-L} as well as with various clinical HIV-1 isolates, and reduce Tat-mediated cytopathicity in infected cells by greater than 75%. The results indicate that anti-HIV-1-Tat-MPC antibodies inhibit viral pathogenesis, possibly by blocking functional determinants of Tat and disrupting autocrine and paracrine actions of secreted Tat protein. This epitope-specific synthetic Tat construct provides a subunit AIDS vaccine for inducing an effective immunoprophylaxis response to reduce progression of HIV infection.

Description of the Preferred Embodiment

We have constructed a synthetic HIV-Tat multiple peptide conjugate constituting the highly conserved cysteine-rich and basic domains by which HIV-Tat protein promotes viral pathogenesis. We have demonstrated that antibodies against this immunogen effectively reduce HIV pathogenesis in vitro. Our general approach overcomes many of the problems associated with immunodominance of non-essential epitopes, viral diversity, and mutational changes in order to design an effective vaccine.

The preferred embodiment of the present invention utilizes three pathogenesis-related functional domains of the HIV-Tat protein: the cysteine-rich Tat_{21-40} and basic Tat_{53-68} domains critical for promoting HIV and the Tat_{9-20} domain of HIV-1 group O.

Consensus-B:

Tat21-40: ACTNCYCKKCCFHCQVCFTT (SEQ ID NO:1)

Tat53-68: RQRRRAHQNSQTHQAS (SEQ ID NO:2)

Consensus-O:

Tat9-20: PPWHHPGSQPQI (SEQ ID NO:3)

The three peptide sequences described above were used to prepare the synthetic construct HIV-1-Tat-MPC, the structure of which is shown in Figure 8. The Tat_{9-20} domain of HIV-1 group O enhances HIV infection in monocytes (Figure 9). Construction of HIV-1-Tat-MPC utilized an Fmoc solid-phase synthesis coupled with conventional solution chemistry as described above. The HIV-1-Tat-MPC was constructed with Tat_{53-68}+Tat_{9-20} as a base peptide and Tat_{21-40} as a linear peptide attached to the tetrameric core template (Figure 8). Both the base peptide Tat_{53-68}+Tat_{9-20} on the core and Tat_{21-40}, which was modified as a linear N-chloroacetyl (Cys-tButhio) derivative, were

purified by HPLC and subjected to MALDI-MS mass spectroscopic analysis to confirm the molecular mass of the peptide species. Mass spectroscopic profiles of purified peptides by MALDI-MS indicated both base peptide Tat_{53-68} + Tat_{9-20} and N-chloroacetyl (Cys-tButhio)-Tat_{21-40} peptide to be highly purified, and the molecular ions observed for each species were consistent with that of the theoretical masses. Final assembly of the HIV-1-Tat-MPC construct involved coupling of purified N-chloroacetyl (Cys-tButhio)-Tat_{21-40} peptide to the cysteine thiol groups on the core containing the base peptide Tat_{53-68} + Tat_{9-20} as described above. The t-Buthio protecting groups were removed and purity of HIV-1-Tat-MPC was established by reverse-phase HPLC (RP-HPLC) on a 4.6 x 25 cm Vydac C18 column with a 0-80% gradient of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. HIV-1-Tat-MPC eluted as a major peak at 55% acetonitrile. A minor peak at 7.6 min, also observed in the reagent blank run, indicated the presence of a trace amount of unrelated product. The fractions eluted in the major peak at 9.353 min retention time were pooled, lyophilized, repurified by HPLC, and stored at -70°C. Homogeneity of this HIV-1-Tat-MPC preparation was further evaluated by SDS-polyacrylamide electrophoresis with immunological characterization by Western blot analysis. HIV-1-Tat-MPC consisted of a predominantly homogeneous species (> 90%) with an apparent molecular mass of 12 kDa that showed strong reactivity with rabbit anti-Tat antiserum. In contrast, purified recombinant Tat used as a positive control exhibited multiple bands, indicating the presence of polymerized products.

Next, the ability of HIV-1-Tat-MPC to induce an effective immune response was tested by immunizing three groups of BALB/c mice with various doses of HIV-1-Tat-MPC by intraperitoneal injections. Serum antibody responses were monitored by ELISA. All three groups of mice immunized with 10-100 ug doses of HIV-1-Tat-MPC developed antibody titers (Fig. 10, panel *a*). The largest immune response was observed in mice immunized with 100 ug of antigen. Peak antibody titer was observed at six weeks (second bleed) for all groups of mice. The antibody titers at six weeks after the initial immunization achieved detectable levels even up to 1:243,000 serum dilutions (Fig. 10, panel *b*).

The binding of anti-HIV-1-Tat-MPC antisera to rTat and the various synthetic peptides present in the construct was determined by ELISA using 96-well plates coated with 10 ug/ml of each antigen. As evident from the data, antisera raised against HIV-1-Tat-MPC showed strong reactivity with rTat as well as with all three Tat peptides. To evaluate the specificity of anti-HIV-1-Tat-MPC antisera, the ability of purified peptides to inhibit binding of HIV-1-Tat-MPC antisera to rTat protein was examined. Preincubation of anti-HIV-1-Tat-MPC antisera with purified peptides in various combinations substantially inhibited the reactivity of anti-HIV-1-Tat-MPC with rTat (Fig. 11, panel *b*), thus confirming the ability of each epitope on the construct to induce an effective immune response.

Cell proliferation in response to Tat or various Tat peptides was evaluated using splenocytes of mice immunized with HIV-1-Tat-MPC. Splenocytes from mice obtained four weeks after the last vaccination were cultured for three days in the presence of rTat, HIV-1-Tat-MPC, or various Tat peptides, and antigen-stimulated cell proliferation

was determined by ^{3}H-thymidine incorporation. Specific responses were observed in spleen cells from mice immunized with HIV-1-Tat-MPC (Figure 12). Tat_{53-68} and Tat_{9-20} peptides induced proliferative responses four-fold, and Tat_{21-40} enhanced proliferation two-fold over control Tat_{41-52} peptide. Recombinant Tat protein used as the positive control also stimulated proliferation by greater than two-fold. Basal levels of proliferation were observed in spleen cells treated with the control peptide or medium only and in cells from unvaccinated control mice.

The ability of anti-HIV-1Tat-MPC antisera to inhibit Tat-stimulated HIV-1 production was evaluated by infection of monocytes in the presence of endogenous or exogenous HIV-Tat protein. Monocytes were infected with HIV_{Ba-L} in the presence or absence of rTat (20 ng/ml) and anti-HIV-1-Tat-MPC antiserum from each mouse at a final dilution of 1:100. The medium containing rTat or anti-HIV-1-Tat-MPC antiserum was replaced every alternate day. On day five, culture media were replaced with medium without rTat or antisera, and the level of virus production was quantitated 24 h later by measuring cell-free p24 antigen by ELISA. The data summarized in Table One reveal that 10 ug or 20 ug of the immunogen elicited effective responses in sera of most animals for neutralizing Tat-induced HIV replication. However, despite the high immune response according to ELISA in mice inoculated with 100 ug of the immunogen, sera from these mice were slightly less effective in neutralization of Tat effects compared to those inoculated with 10 or 20 ug of the antigen (Table One; $P < 0.01$ vs. 20 ug and $P < 0.05$ vs. 10 ug).

HIV_{Ba-L}-associated cytopathic effects were observed to be reduced by 60-75% in monocytes treated with anti-HIV-1-Tat-MPC antisera. Importantly, the neutralizing anti-HIV-1-Tat-MPC antisera were also able to inhibit Tat-mediated induction of HIV replication by 15-67% in monocytes infected with a variety of clinical isolates (Table Two).

Figure 13 depicts a possible mechanism by which anti-HIV-1-Tat-MPC inhibits Tat-induced HIV replication in monocytes. Extracellular Tat protein produced by HIV-infected cells is believed to act on cells both in autocrine and paracrine fashion. Tat activates normal cells for productive HIV infection and then promotes virus replication in HIV-infected cells. It is likely that anti-HIV-1-Tat-MPC antibodies target key functional determinants and disrupt the autocrine and paracrine actions induced by secreted Tat protein.

We describe a novel synthetic multiple-peptide conjugate system constituting three functional peptide sequences of the HIV-1 Tat protein in a single, homogenous immunogen. All three peptides constituting the construct induced an effective immune response in mice and generated antisera that efficiently inhibited Tat-induced pathogenesis in monocytes infected with HIV_{Ba-L} as well as various clinical HIV-1 isolates. Importantly, HIV-1-Tat-MPC alone was sufficient to induce an effective immune response without being conjugated to a carrier protein. Thus,

this construct represents the first successful model of a synthetic immunogen that serves as a basis for designing a multi-epitope subunit vaccine for AIDS.

The three peptides used to generate the HIV-1-Tat-MPC construct were all immunogenic, and antibodies raised in mice against the HIV-1-Tat-MPC reacted with recombinant Tat protein from which the peptide sequences had been deduced. Furthermore, Tat-induced pathogenesis was inhibited by antisera against HIV-1-Tat-MPC, supporting roles for Tat_{21-40}, Tat_{53-68}, and Tat_{9-20} epitopes in viral activation by infected cells. Therefore, this HIV-1-Tat-MPC containing conserved functional domains from group M of HIV-1, as well as a new functional sequence from the newly discovered HIV-1 strain group O, serves as an immunogen for various subtypes of HIV-1 infection. Vaccination of mice with low doses (10 to 20 ug) of the HIV-1-Tat-MPC construct elicited antibodies that efficiently inhibited Tat effects; 100 ug-injections did not enhance efficacy and even appeared to significantly decrease effectiveness in supporting HIV-1 viral production. It is practical to optimize the minimal dose of the HIV-1-Tat-MPC to induce maximal immune responses in mice and other animal species.

Our findings provide a new approach to developing effective and safe subunit vaccines, especially when a conventional vaccine may fail to induce an effective immune response due to problems associated with immunodominance, viral diversity, and mutational instability. The fact that HIV-1-Tat-MPC can be readily customized by replacing peptides present on the construct with desired peptides from other viral antigens (such as gp120, gp41 or gag regions as needed) provides a versatile tool for generating synthetic subunit vaccines. This flexibility is a significant advantage over conventional vaccines, especially for heterogeneous viruses such as HIV. Thus, our observations constitute a major advance in HIV research by providing a completely novel strategy to develop a synthetic AIDS vaccine based on a new type of multiple peptide antigen constituting a homogeneous entity with multiple epitopes. Because HIV-1-Tat-MPC is a purely synthetic molecule that does not contain a live or inactivated virus, it conveys no risk of viral transmission to recipients. These advances form the basis for immunization of primates and humans.

Example 18

HIV-1-Tat protein. The HIV-1-Tat protein used in these studies was obtained as a gift from Dr. John Brady (National Cancer Institute, National Institutes of Health) or through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergies and Infectious Disease, National Institutes of Health, from Dr. Andrew Rice or Dr. John N. Brady (Lafrenie et al., *J. Immunol.*, 156:1638 (1996)). HIV-1 Tat was dissolved at 10 ug/ml in 1,000X

treatment buffer (PBS containing 1 mg/ml BSA, 0.1 mM dithiothreitol) and frozen in aliquots at -80°C. Tat preparations were screened and found to be negative for endotoxin contamination.

Synthesis of Tat peptides and multiple peptide construct. Tat peptides were synthesized by solid-phase synthesis on an Applied Biosystems Peptide synthesizer Model 430A (Foster City, CA). Peptide identities were confirmed by amino acid compositional analysis and plasma desorption mass spectroscopic analysis. The HIV-Tat-1-MPC was prepared according to the above-described procedure.

Immunization of mice with HIV-1-Tat-MPC. Groups of five BALB/c mice (6-8 weeks old) were inoculated three times at three-week intervals with 10 to 100 ug of the HIV-1-Tat-MPC construct (Strategic BioSolutions, Newark, DE). Constructs were mixed with an equal volume of complete Freund's adjuvant for primary injections and incomplete Freund's adjuvant for secondary and tertiary injections. Sera were prepared from blood taken 15 days after each injection.

Serological analysis. Enzyme-linked immunosorbent assay (ELISA) was performed using HIV-1-Tat-MPC coated on 96-well microtiter plates (Costar, Cambridge, MA) at a concentration of 1 ug/ml (Strategic BioSolutions). Antibody titers were expressed as optical density (OD) values measured at 650 nm on 1,000-fold diluted antiserum specimens.

Monocyte isolation and infection with HIV-1. Monocytes were isolated from peripheral blood mononuclear cells (PBMC) of donors seronegative for HIV and hepatitis by leukapheresis and purification by countercurrent centrifugal elutriation (Lafrenie et al., *J. Immunol.*, 156:1638 (1996)). In some experiments, monocytes were treated with HIV-1-Tat protein. Primary monocytes cultured for 5 days were exposed to HIV-1_{Ba-L}, a monocytotrophic HIV strain (Advanced Biotechnologies, Inc., Columbia, MD) at a multiplicity of infection of 0.005 infectious virus particles/target cell, or with various clinical HIV-1 isolates.

Determination of virus production. The levels of cell-free HIV-1 in culture supernatants were determined using DuPont NEN p24 ELISA kits for HIV-1.

Cell proliferation assay. Spleens from vaccinated or non-vaccinated (control) mice were aseptically excised and used to prepare single-cell suspensions in RPMI-1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 5% heat-inactivated FCS, 100 units/ml penicillin, and 100 ug/ml streptomycin. Cell viability was >90% as determined by trypan blue exclusion. Splenocytes (2 X 10^5 cells) were cultured in round-bottom 96-well tissue culture plates (Costar) with 10 ug/ml rTat, various Tat peptides, or medium alone and incubated in a 5% CO_2 humidified incubator at 37°C for 96 h and pulsed with 1 uCi ^{3}H-thymidine/well during the last 4 h. Cells were

harvested and ^{3}H-thymidine incorporation was determined by liquid scintillation counting. All cultures were performed in triplicate.

Detection of rTat and HIV-1-Tat-MPC by Western blot analysis. The lyophilized rTat protein or HIV-1-Tat-MPC were resuspended in SDS-Laemmli loading buffer [500 mM Tris-HCl, pH 6.8, 10% SDS, 0.01% bromophenol blue, 20% glycerol], reduced with 1% -mercaptoethanol, heated for 2 min at 95^{0}C, loaded, and electrophoresed on a 8-16% Tris-glycine gradient polyacrylamide gel (Novex, San Diego, CA) in SDS running buffer [25 mM Tris-HCl, pH 8.3, 192 mM glycine, 10% SDS]. After electrophoresis, the rHIV-1-Tat protein and HIV-1-Tat-MPC were transferred onto 0.45-uM nitrocellulose in a buffer containing 25 mM Tris-HCl [pH 8.3, 192 mM glycine, 20% methanol] and blocked with 50 mM Tris-HCl [pH 7.5, 150 mM NaCl, 0.2% Tween-20 (TBST)] containing 5% nonfat dry milk for at least 1 h. The blots were washed three times with TBST and then incubated for 1 h with rabbit anti-Tat antibody followed by horseradish peroxidase (HRP)-conjugated protein A (Pierce Chemicals, Rockford, IL; 1:3,000 dilution in TBST containing 5% nonfat dry milk) and developed with SuperSignal chemiluminescence enhancer solution (Pierce). The bands were visualized by exposure of blots to X-ray film.

Table One

Effect of antisera against HIV-1-Tat-MPC on Tat-induced HIV-1 production by monocytes

Mouse Number	Dose of Immunogen	Inhibition of HIV infection (% of Control) Bleed #1	Bleed #2	Mean±SEM Bleed # 1	Bleed #2
1.1	10 ug	0	2		
1.2	10 ug	54	54		
1.3	10 ug	68	100	54.4±14.4	64.2±18.1
1.4	10 ug	66	100	($P<0.02$)	($P<0.03$)
1.5	10 ug	84	65		
2.1	20 ug	84	74		
2.2	20 ug	69	81		
2.3	20 ug	78	99	46.2±19.0	86.0±5.6
2.4	20 ug	0	100	($P=0.07$)	($P<0.0001$)
2.5	20 ug	0	76		
3.1	100 ug	38	23		
3.2	100 ug	35	82		
3.3	100 ug	0	33	42.2±13.2	46.2±10.0
3.4	100 ug	79	48	($P<0.04$)	($P<0.01$)
3.5	100 ug	59	45		

Monocytes (2 x 10^5 cells) cultured in 96-well plates were treated with HIV-Tat (20 ng/ml) in the presence or absence of mouse antisera against HIV-1-Tat-MPC (pooled 20 ug immunizations, 1:100 dilution) 2 h prior to HIV infection at an MOI of 0.005. After a 2 h exposure, virus was removed, and the culture medium was replaced with fresh medium with or without rTat in the presence or absence of antisera against HIV-1-Tat-MPC (1:100). On day 5, culture medium was replaced with fresh medium with no additions. After 24 h, culture fluid was harvested for p24 determination. The data are presented as inhibition of HIV production measured as cell-free p24 relative to HIV-infected monocytes treated with rTat in the absence of antisera. *P* values indicate statistical significance of difference from 0% inhibition by 2-tail Student's t-test.

Table Two

Effect of antisera against HIV-1-Tat-MPC on Tat-induced HIV-1 production by monocytes infected with various clinical HIV-1 isolates

HIV-1 isolate	Date of collection	Coreceptor usage	p24 (ng/ml) -Tat	-Tat +anti-MPC antisera	p24 (ng/ml) +Tat	+Tat +anti-MPC antisera
Rapid progressors:						
5084	Oct. 86	R5	8.3±2.7	4.7±1.0 (43)	11.8±1.8	5.0±0.6 (58)
5108	Oct. 85	ND	1.9±0.7	1.4±0.2 (30)	2.6±0.3	1.9±0.5 (26)
5157	Mar. 84	R5	1.8±0.4	2.0±0.6 (-)	2.7±0.3	1.6±0.5 (40)
Late progressors:						
5048	Mar. 87	ND	3.8±0.5	3.0±0.4 (20)	6.9±0.7	3.2±0.5 (54)
5160	Jul. 84	R5	2.1±0.3	1.0±0.1 (53)	3.3±0.2	1.8±0.4 (47)
5073	May 89	R5	0.7±0.1	0.6±0.1 (15)	1.5±0.2	0.7±0.1 (51)
Long term non-progressors:						
5096	Mar. 89	ND	10.1±1.0	7.2±0.6 (29)	15.7±0.2	6.7±0.4 (57)
5148	Apr. 83	R5	1.3±0.2	1.06±0.3 (16)	1.9±0.4	0.6±0.10 (67)
5155	May 95	R5	9.3±0.5	4.6±0.5 (51)	17.7±0.2	8.1±0.9 (54)

Monocytes (2×10^5 cells) cultured in 96-well plates were treated with HIV-Tat (20 ng/ml) in the presence or absence of mouse antisera against HIV-1-Tat-MPC (pooled 20 ug immunizations, 1:100 dilution) 2 h prior to HIV infection with various HIV-1 isolates (40,000 RT/10^6 cells). After a 2 h exposure, virus was removed, and the culture medium was replaced with fresh medium with or without rTat in the presence or absence of antisera against HIV-1-Tat-MPC (1:100). On day 5, culture medium was replaced with fresh medium with no additions. After 24 h, culture fluid was harvested for p24 determination. The p24 values are expressed as mean±SEM of three determinations. Values in parentheses represent percent inhibition of HIV production by anti-HIV-1-Tat-MPC antisera. ND: not determined.

Example 19

Effect of anti-HIV-1-Tat-MPC antibody on uninfected or HIV-1-infected monocyte migration in response to HIV-1 Tat protein. Uninfected or HIV-infected monocytes were suspended at 2 X 10^6 cells/ml. HIV-1 Tat (20 ng/ml) was placed in the lower chamber of a chemotactic chamber in the presence of normal mouse serum or mouse anti-HIV-1-Tat-MPC antiserum (1:100), covered with a polycarbonate filter, and 50 ul of the uninfected or HIV-1-infected monocyte suspension was placed in the upper chamber. The chamber was incubated for 3 h at 37°C and then the filters were removed and stained with Diff-Quick stain. The number of cells attached to the underside of the filter was determined by high power microscope field in triplicate for each well. Anti-HIV-Tat-MPC antibody substantially inhibited (> 70%) HIV-1 Tat-induced migration of uninfected and HIV-infected monocytes.

Example 20

Effect of anti-HIV-1-Tat-MPC on invasion of uninfected or HIV-1-infected monocytes through basement membrane matrix (Matrigel). Uninfected or HIV-1-infected monocytes were suspended at 2 X 10^6 cells/ml. HIV-1-Tat (20 ng/ml) was placed in the lower chamber in the presence of normal mouse serum (1:100) or mouse anti-HIV-1-Tat-MPC antiserum (1:100), covered with a Matrigel-coated polycarbonate filter, and 50 ul of the uninfected or HIV-1-infected monocyte suspension was placed in the upper chamber. The chamber was incubated for 12 h at 37°C, and then the filters were removed and stained. The number of cells attached to the underside of the filter was determined by high power microscope field in triplicate for each well. Preincubation of HIV-1-Tat with anti-HIV-1-Tat-MPC antibody inhibited monocyte invasion across the basement membrane matrix by greater than 60%.

Inventorship

Please note that the inventors of each of the separate parts of this application are as follows:

Part I: Subhash Dhawan, Robert A. Boykins and Kenneth M. Yamada;

Part II: Robert A. Boykins, Manju B. Joshi, Chiang Syin, Subhash Dhawan and Hira L. Nakhasi; and

Part III: Subhash Dhawan and Robert A. Boykins.

WHAT IS CLAIMED IS:

1. An isolated or purified peptide comprising a Tat functional domain, said peptide comprising a portion of the Tat protein selected from the group consisiting of the portion from amino acids 21-40 of the Tat protein, the portion from amino acids 53-68 of the Tat protein and the portion from amino acids 9-20 of the Tat protein.

2. The isolated or purified peptide of Claim 1, wherein said isolated or purified peptide has a sequence selected from the group consisting of SEQ ID NOs 1-21.

3. The isolated or purified peptide of Claim 1, wherein said peptide contains a dominant-negative mutation.

4. An isolated or purified peptide having a sequence selected from the group consisting of SEQ ID NOs. 22-66.

5. An isolated or purified peptide having a sequence selected from the group consisting of:

(a) the sequence of SEQ ID NO: 1 wherein the A (Alanine) at position 1 of the peptide has been substituted with an amino acid selected from the group consisting of cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(b) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 2 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(c) the sequence of SEQ ID NO: 1 wherein the T (threonine) at position 3 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine;

(d) the sequence of SEQ ID NO: 1 wherein the N (asparagine) at position 4 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, thereonine, valine, tryptophan, and tyrosine;

(e) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 5 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(f) the sequence of SEQ ID NO: 1 wherein the Y (tyrosine) at position 6 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, and tryptophan;

(g) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 7 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(h) the sequence of SEQ ID NO: 1 wherein the K (lysine) at position 8 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(i) the sequence of SEQ ID NO: 1 wherein the K (lysine) at position 9 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(j) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 10 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(k) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 11 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(l) the sequence of SEQ ID NO: 1 wherein the F (phenylalanine) at position 12 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(m) the sequence of SEQ ID NO: 1 wherein the H (histidine) at position 13 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(n) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 14 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine,

histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(o) the sequence of SEQ ID NO: 1 wherein the Q (glutamine) at position 15 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(p) the sequence of SEQ ID NO: 1 wherein the V (valine) at position 16 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, tryptophan, and tyrosine;

(q) the sequence of SEQ ID NO: 1 wherein the C (Cysteine) at position 17 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(r) the sequence of SEQ ID NO: 1 wherein the F (phenylalanine) at position 18 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine;

(s) the sequence of SEQ ID NO: 1 wherein the T (threonine) at position 19 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine; and

(t) the sequence of SEQ ID NO: 1 wherein the T (threonine) at position 20 of the peptide has been substituted with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, valine, tryptophan, and tyrosine.

6. An isolated or purified peptide that is at least 70 percent identical to a peptide having a sequence selected from the group consisting of SEQ ID NOs: 1-21 wherein percent identity is determined by FASTA or BLAST using default opening and gap penalties and a PAM scording matrix.

7. A nucleic acid encoding the peptide of any of Claims 1-6.

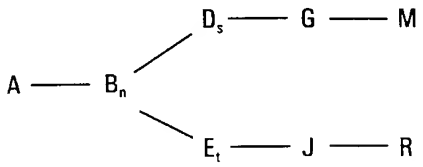
8. A peptide of any of Claims 1-6 or a nucleic acid encoding the peptide of any of Claims 1-6 for use in reducing the activity of Tat in an individual infected with HIV or inducing an immune response against the Tat protein.

9. A method of reducing the activity of Tat in an individual infected with HIV or inducing an immune response in an individual against the Tat protein of HIV comprising the step of administering the peptide of any of

Claims 1-6 or a nucleic acid encoding the peptide of any of Claims 1-6 to said individual in an amount sufficient to reduce the activity of Tat or induce an immune response against the Tat protein.

10. An isolated or purified complex comprising the peptide of any of Claims 1-6 and an HIV LTR.

11. A compound having the structure:



wherein:

A is absent or an amino acid;

B is an amino acid;

D is absent or an amino acid;

E is absent or an amino acid;

G is an amino acid;

J is an amino acid;

M is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

R is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

n is 1-10;

s is 0 when D is absent or is 1-20 when D is present; and

t is 0 when E is absent or is 1-20 when E is present.

12. The compound of Claim 11, wherein D and E are absent.

13. The compound of Claim 12, wherein A comprises an amino acid having a functional group thereon which permits attachment to a solid support.

14. The compound of Claim 13, wherein A is serine.

15. The compound of Claim 14, wherein said serine is Ser(Trt).

16. The compound of Claim 15, wherein B is an amino acid having functional groups which permit two amino acids to be attached thereto.

17. The compound of Claim 16, wherein B is lysine.

18. The compound of Claim 17, wherein G and J are lysines.

19. The compound of Claim 18, wherein the ε amino groups of G and J are protected with t-Boc.

20. The compound of Claim 19, further comprising a peptide linked to said first reactive groups of M and R.

21. The compound of Claim 20, wherein said peptide comprises at least one antigenic site.

22. The compound of Claim 21, further comprising a peptide linked to said second reactive groups of M and R.

23. The compound of Claim 22, wherein the peptide linked to said second reactive groups of M and R comprise at least one antigenic site.

24. The compound of Claim 18, wherein M and R are cysteines and wherein the amino and SH groups of said cysteines are protected with blocking groups which permit amino acids or peptides to be selectively attached thereto.

25. The compound of Claim 24, wherein the amino groups of said cysteines are protected with Fmoc.

26. The compound of Claim 25, wherein the SH groups of said cysteines are protected with Trt.

27. The compound of Claim 23, wherein M and R are cysteines, said first reactive groups are the amino groups of said cysteines, and said second reactive groups are the thiol groups of said cysteines.

28. The compound of Claim 27, wherein the peptide attached to the amino group of M is the same as the peptide attached to the amino group of R.

29. The compound of Claim 27, wherein the peptide attached to the amino group of M is different than the peptide attached to the amino group of R.

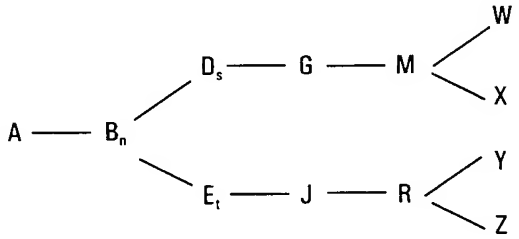
30. The compound of Claim 27, wherein the peptide attached to the thiol group of M is the same as the peptide attached to the thiol group of R.

31. The compound of Claim 27, wherein the peptide attached to the thiol group of M is different than the peptide attached to the thiol group of R.

32. The compound of Claim 21, wherein n is 1-5, s is 1-10, and t is 1-10.

33. The compound of Claim 21, wherein n is 1, s is 0, and t is 0.

34. A compound having the structure:



wherein:

A is absent or an amino acid;

B is an amino acid;

D is absent or an amino acid;

E is absent or an amino acid;

G is an amino acid;

J is an amino acid;

M is an amino acid having two reactive groups thereon which can be attached to an amino acid;

R is an amino acid having two reactive groups thereon which can be attached to an amino acid;

W is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

X is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

Y is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

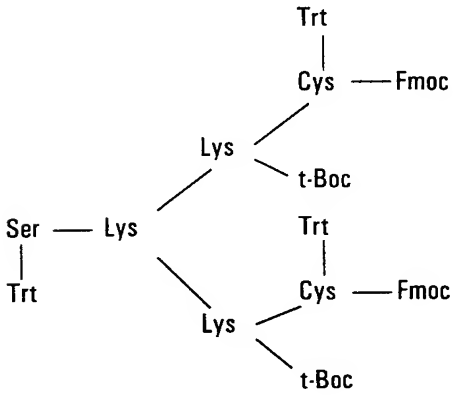
Z is an amino acid having a first reactive group and a second reactive group thereon, wherein said first reactive group and said second reactive group are capable of selectively being attached to an amino acid or peptide;

n is 1-10;

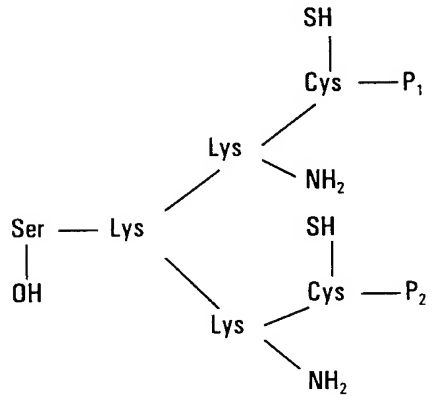
s is 0 when D is absent or is 1-20 when D is present; and

t is 0 when E is absent or is 1-20 when E is present.

35. A compound having the structure:



36. A compound having the structure:



wherein P_1 and P_2 are peptides.

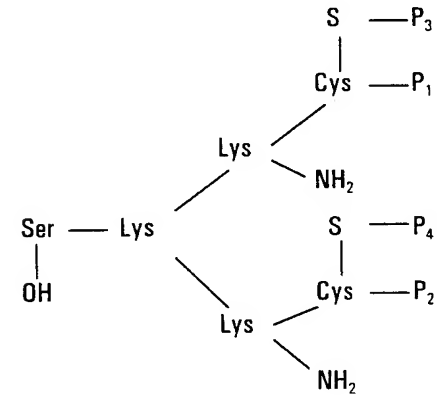
37. The compound of Claim 36, wherein P_1 and P_2 comprise at least one antigenic site.

38. The compound of Claim 37, wherein P_1 and P_2 are selected from the group consisting of malarial and HIV peptides.

39. The compound of Claim 38, wherein at least one of said malarial peptides comprises a peptide selecting from the group consisting of SEQ ID NOs: 81-85.

40. The compound of Claim 38, wherein at least one of said HIV peptides comprises a peptide selected from the group consisting of SEQ ID NOS.: 1-21.

41. A compound having the structure:



wherein P_1, P_2, P_3 and P_4 are peptides.

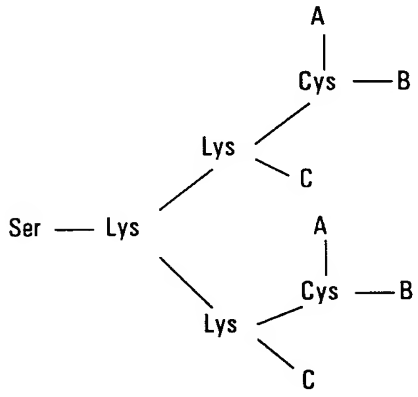
42. The compound of Claim 41, wherein P_1, P_2, P_3 and P_4 comprise at least one antigenic site.

43. The compound of Claim 42, wherein P_1, P_2, P_3 and P_4 are selected from the group consisting of malarial and HIV peptides.

44. The compound of Claim 43, wherein at least one of said malarial peptides comprises a peptide selected from the group consisting of SEQ ID NOs: 81-85.

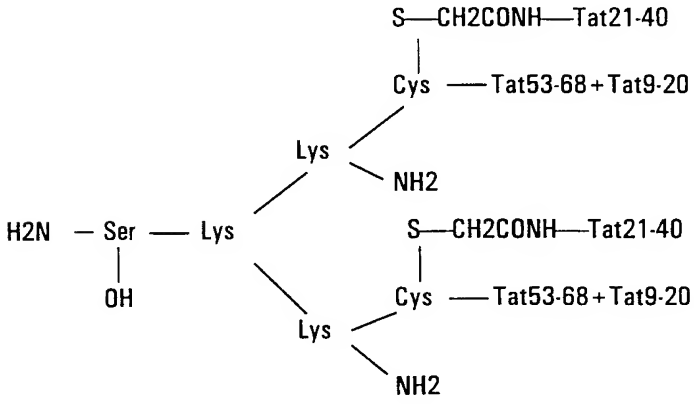
45. The compound of Claim 43, wherein at least one of said HIV peptides comprises a peptide selected from the group consisting of SEQ ID NOs: 1-21.

46. A compound having the structure:



wherein A is a blocking group which can be removed without removing B or C, B is a blocking group which can be removed without removing A or C, and C is a blocking group which can be removed without removing A or B.

47. HIV-1-Tat-MPC having the structure:



48. A method of making a multiple peptide conjugate comprising the steps of:

obtaining a core template having at least two branches, said at least two branches being separated from one another by at least four carbons, wherein each of said branches have a first reactive group and a second reactive group thereon;

synthesizing a peptide on said first reactive groups on said at least two branches; and

attaching a pre-synthesized peptide on said second reactive groups on said at least two branches.

49. The method of Claim 48, wherein one or more of the amino acids used in said synthesizing step are blocked with agents which reduce steric hindrance.

50. The method of Claim 49, wherein said agents which reduce steric hindrance comprise N-[2-hydroxy-4-methoxybenzyl] groups.

51. The method of Claim 49, further comprising blocking the thiol groups of any cysteine residues used in said synthesizing step or present in said pre-synthesized peptide with an agent which prevents said thiol groups from being reactive.

52. The method of Claim 51, wherein said agent which prevents said thiol groups from being reactive is a tert-butyl group.

53. The method of Claim 52, further comprising removing said agent which prvents said thiol groups from being reactive after said attaching step.

54. The method of Claim 49, further comprising removing said agents which reduce steric hindrance after said attaching step.

55. The method of Claim 48, wherein said synthesizing step is performed in the presence of DMSO.

56. The method of Claim 48, further comprising removing a first blocking group from said first reactive group prior to attaching said amino acid or peptide to said first reactive group under conditions in which a second blocking group is not removed from said second reactive group.

57. The method of Claim 56, wherein said first reactive group comprises the amino group of cysteine and said second reactive group comprises the thiol group of cysteine.

58. The method of Claim 57, wherein said first blocking group is removable with base and said second blocking group is removable with acid.

59. The method of Claim 58, wherein said first blocking group comprises Fmoc and said second blocking group comprises Trt.

60. The method of Claim 48, wherein said core template is selected from the group consisting of the compound of Claim 1, the compound of Claim 24, and the compound of Claim 25.

61. A compound of any of Claims 11-47 for use in inducing an immune response or for use as an intermediate in making a compound for use in inducing an immune response.

62. A method of inducing an immune response in an individual comprising the step of administering a compound of any of Claims 11-47 or a compound made from an intermediate that is a compound of any of Claims 11-47 to said individual in a sufficient amount to induce an immune response.

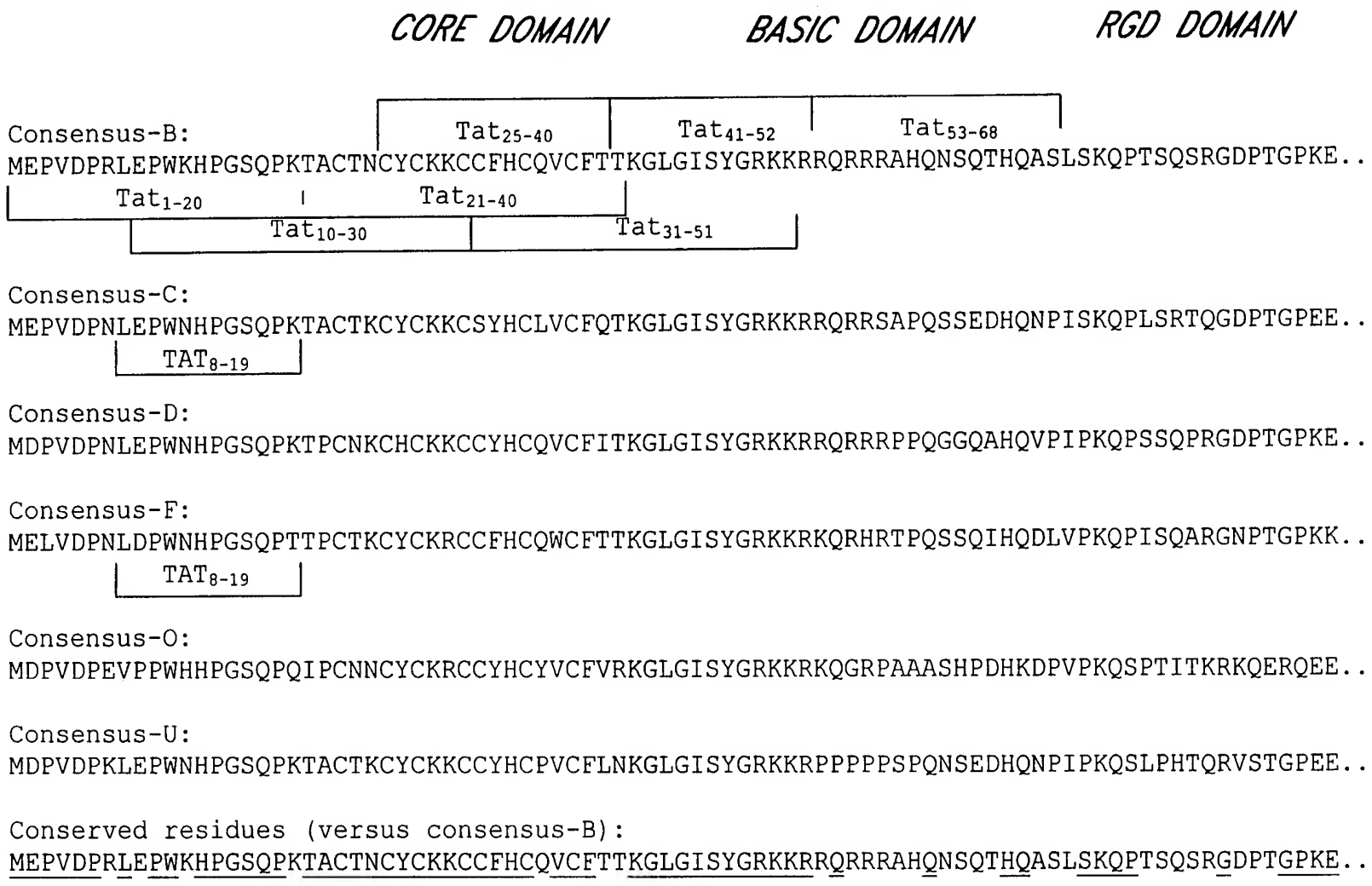
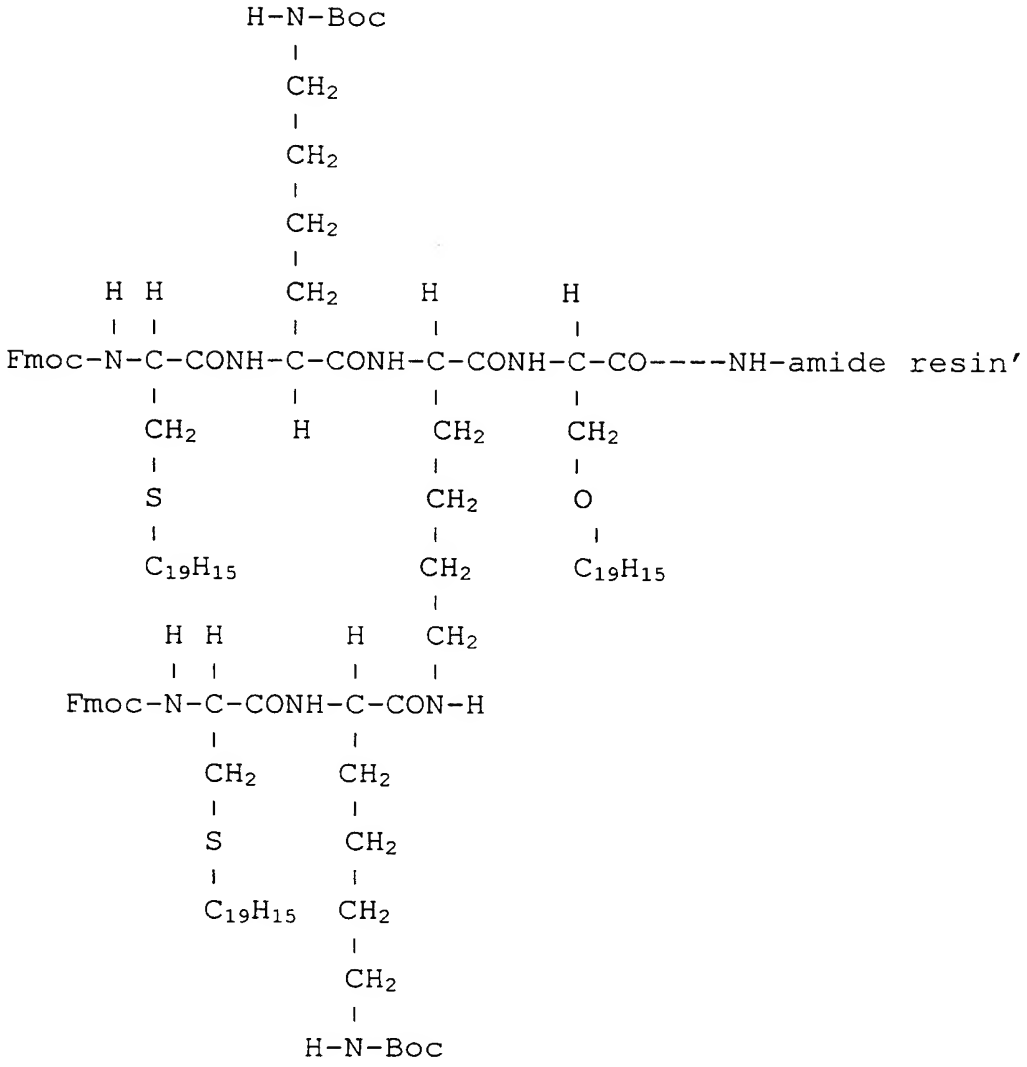

CORE DOMAIN
BASIC DOMAIN
RGD DOMAIN
Consensus-B:
Tat25-40
Tat41-52
Tat53-68
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFTTKGLGISYGRKKRRQRRRAHQNSQTHQASLSKQPTSQSRGDPTGPKE..
Tat1-20
Tat21-40
Tat10-30
Tat31-51
Consensus-C:
MEPVDPNLEPWNHPGSQPKTACTKCYCKKCSYHCLVCFQTKGLGISYGRKKRRQRRSAPQSSEDHQNPISKQPLSRTQGDPTGPEE..
TAT8-19
Consensus-D:
MDPVDPNLEPWNHPGSQPKTPCNKCHCKKCCYHCQVCFITKGLGISYGRKKRRQRRRPPQGGQAHQVPIPKQPSSQPRGDPTGPKE..
Consensus-F:
MELVDPNLDPWNHPGSQPTTPCTKCYCKRCCFHCQWCFTTKGLGISYGRKKRKQRHRTPQSSQIHQDLVPKQPISQARGNPTGPKK..
TAT8-19
Consensus-O:
MDPVDPEVPPWHHPGSQPQIPCNNCYCKRCCYHCYVCFVRKGLGISYGRKKRKQGRPAAASHPDHKDPVPKQSPTITKRKQERQEE..
Consensus-U:
MDPVDPKLEPWNHPGSQPKTACTKCYCKKCCYHCPVCFLNKGLGISYGRKKRPPPPPSPQNSEDHQNPIPKQSLPHTQRVSTGPEE..
Conserved residues (versus consensus-B):
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFTTKGLGISYGRKKRRQRRRAHQNSQTHQASLSKQPTSQSRGDPTGPKE..

FIG. 1

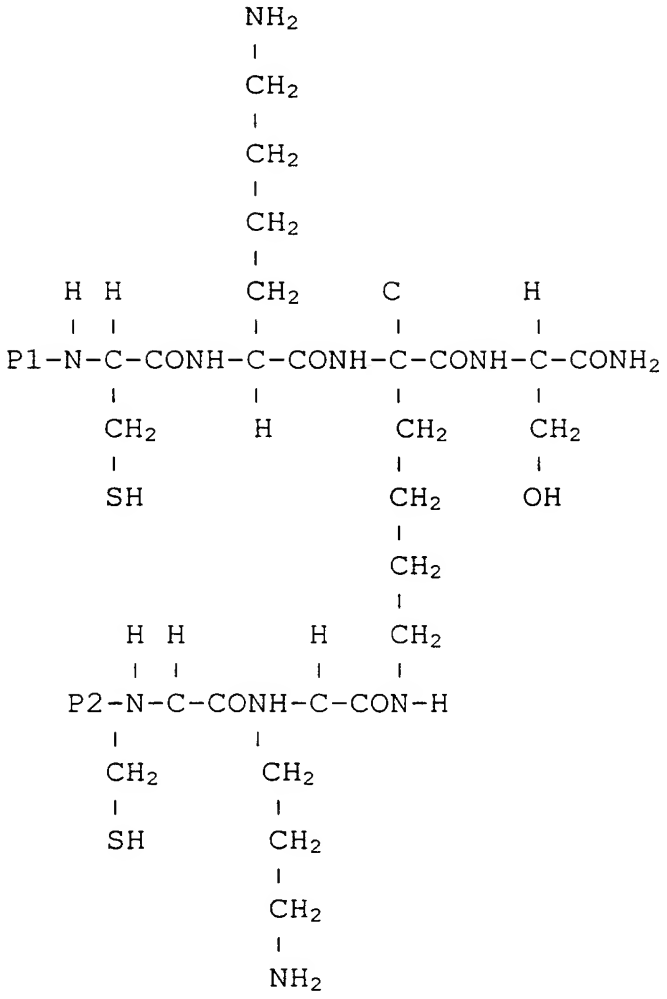
CORE TEMPLATE



C-terminal may also attach to acid linker
Trityl-$C_{19}H_{15}$
Amide resin - Rink amide MBHA

FIG. 2

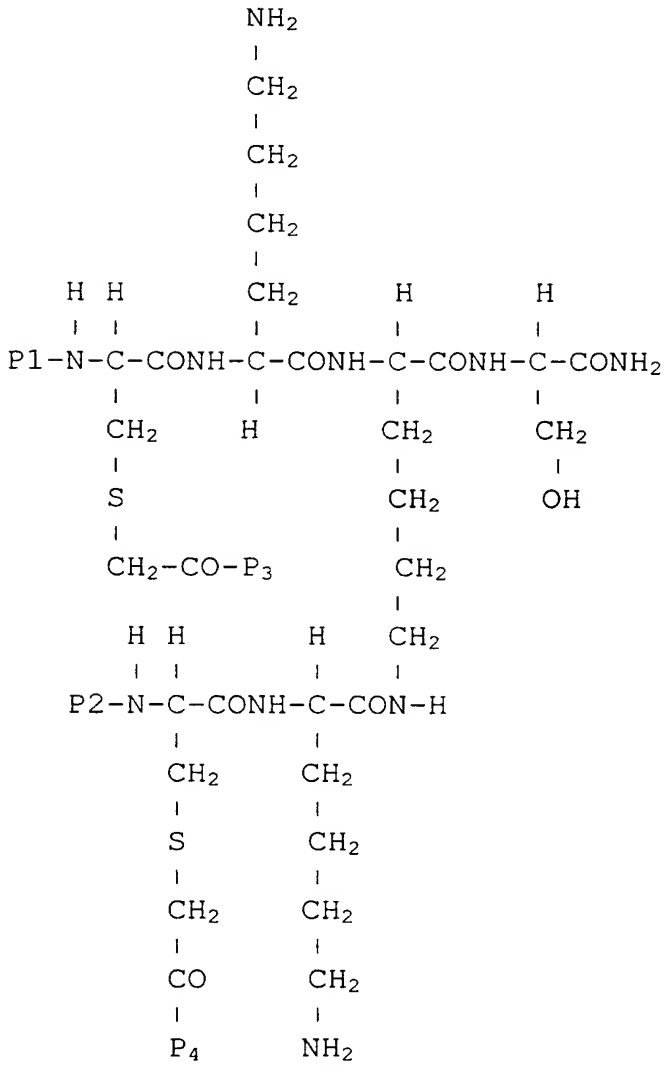
BASE PEPTIDE



P1, P2 - peptides

FIG. 3

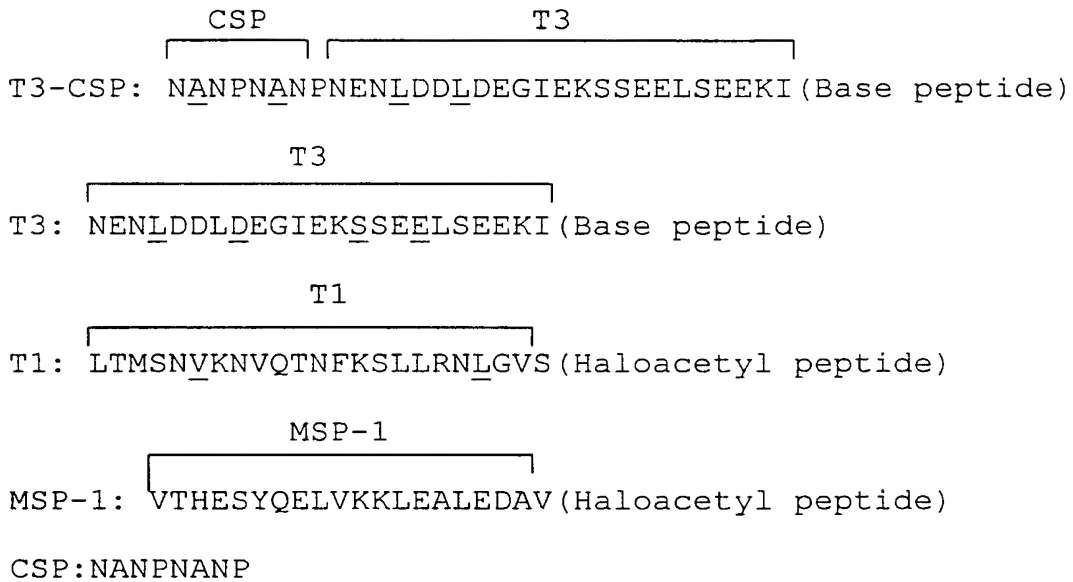
MULTIPLE PEPTIDE CONJUGATE



P1,P2,P3,P4 - Peptides

FIG.4

A. **Malaria Peptide Sequences**



B. **HIV-1-Tat Peptide Sequences**

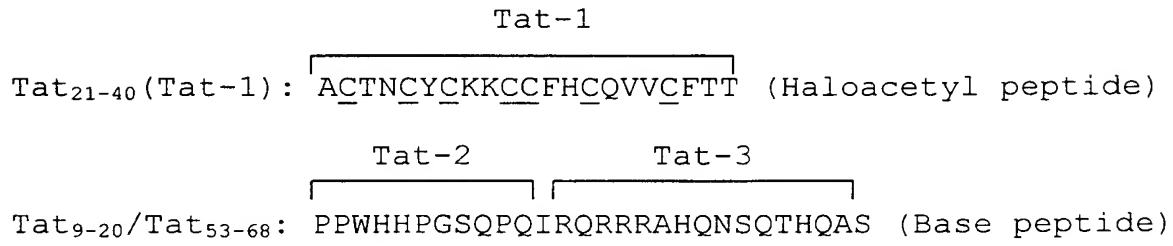


FIG.5

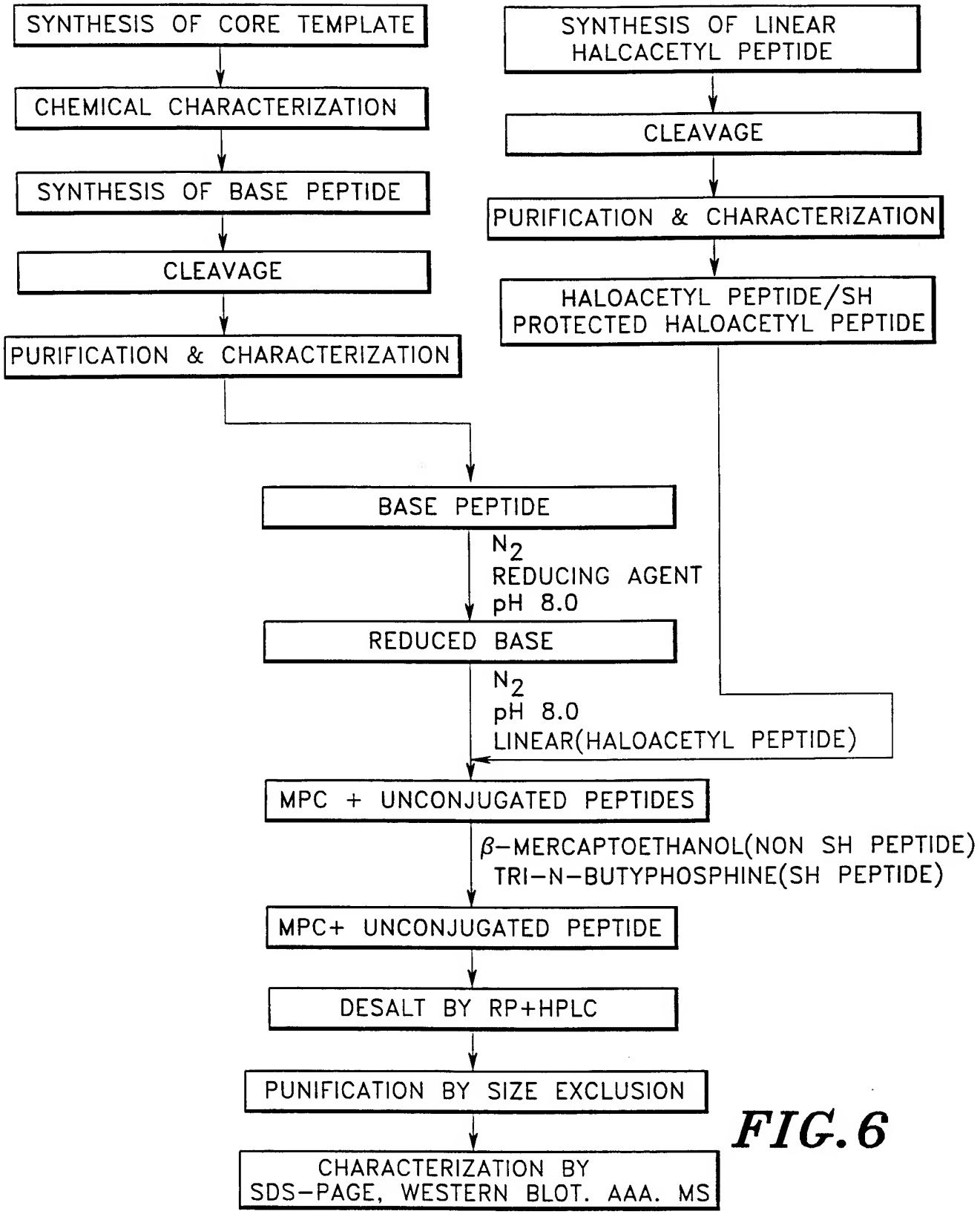

SYNTHESIS OF CORE TEMPLATE
CHEMICAL CHARACTERIZATION
SYNTHESIS OF BASE PEPTIDE
CLEAVAGE
PURIFICATION & CHARACTERIZATION
SYNTHESIS OF LINEAR HALCACETYL PEPTIDE
CLEAVAGE
PURIFICATION & CHARACTERIZATION
HALOACETYL PEPTIDE/SH PROTECTED HALOACETYL PEPTIDE
BASE PEPTIDE
N_2
REDUCING AGENT
pH 8.0
REDUCED BASE
N_2
pH 8.0
LINEAR(HALOACETYL PEPTIDE)
MPC + UNCONJUGATED PEPTIDES
β-MERCAPTOETHANOL(NON SH PEPTIDE)
TRI-N-BUTYPHOSPHINE(SH PEPTIDE)
MPC+ UNCONJUGATED PEPTIDE
DESALT BY RP+HPLC
PUNIFICATION BY SIZE EXCLUSION
CHARACTERIZATION BY SDS-PAGE, WESTERN BLOT. AAA. MS

FIG. 6

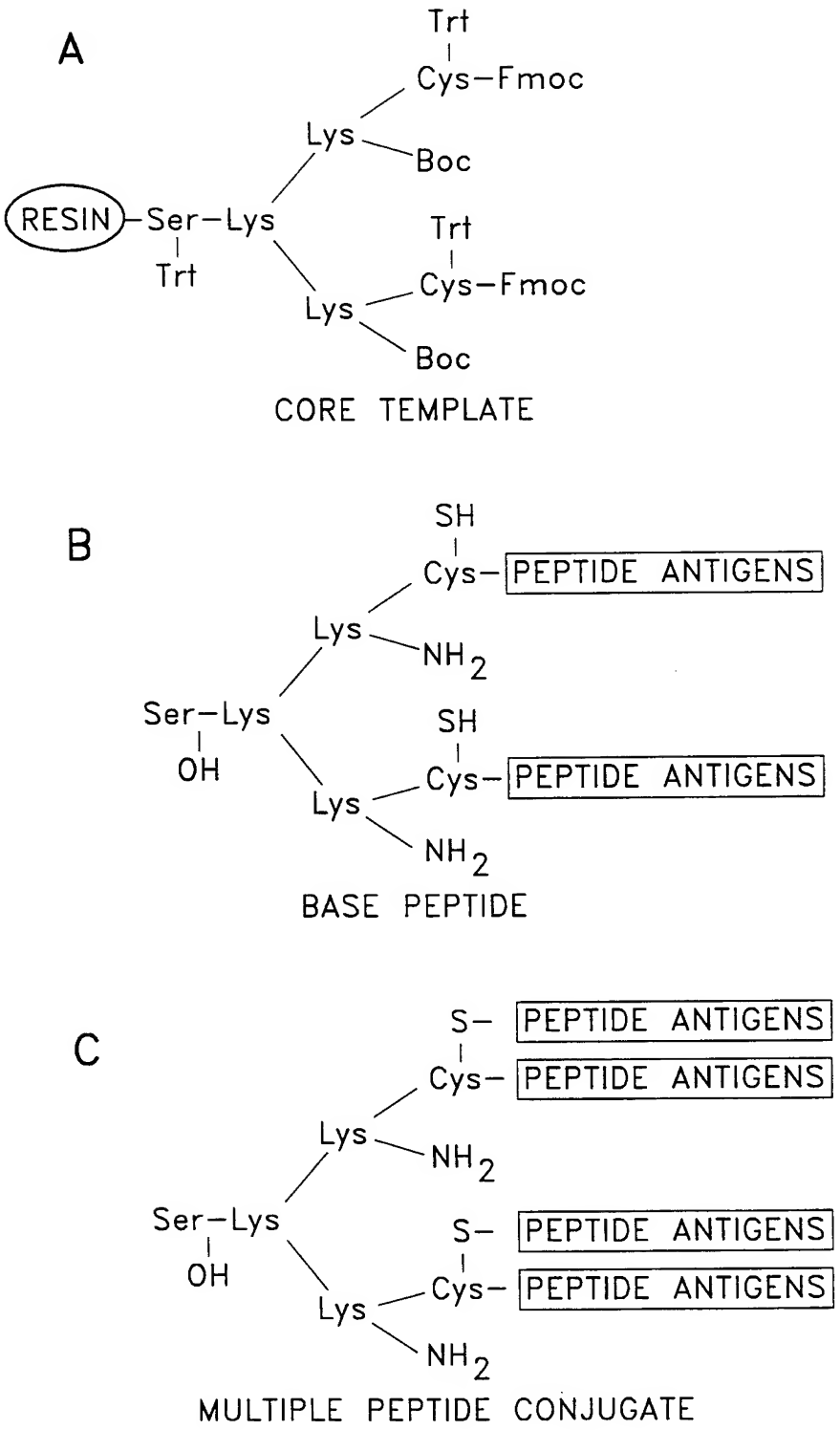

A
Trt
Cys–Fmoc
Lys
Boc
RESIN–Ser–Lys
Trt
Trt
Cys–Fmoc
Lys
Boc
CORE TEMPLATE
B
SH
Cys–PEPTIDE ANTIGENS
Lys
NH2
Ser–Lys
OH
SH
Cys–PEPTIDE ANTIGENS
Lys
NH2
BASE PEPTIDE
C
S– PEPTIDE ANTIGENS
Cys– PEPTIDE ANTIGENS
Lys
NH2
Ser–Lys
OH
S– PEPTIDE ANTIGENS
Cys– PEPTIDE ANTIGENS
Lys
NH2
MULTIPLE PEPTIDE CONJUGATE

FIG. 7

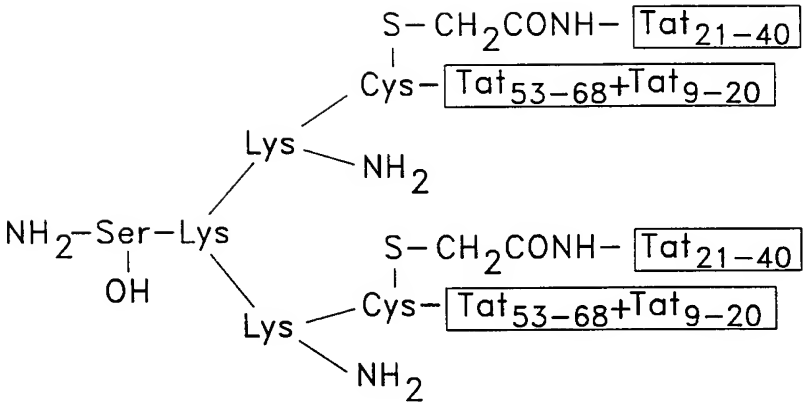

S-CH2CONH-Tat21-40
Cys-Tat53-68+Tat9-20
Lys
NH2
NH2-Ser-Lys
OH
S-CH2CONH-Tat21-40
Cys-Tat53-68+Tat9-20
Lys
NH2

FIG. 8

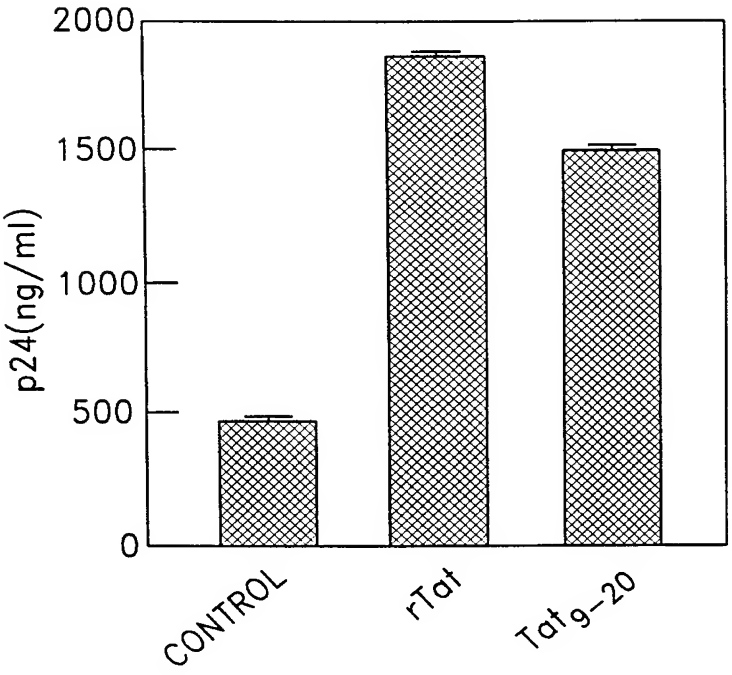


FIG. 9

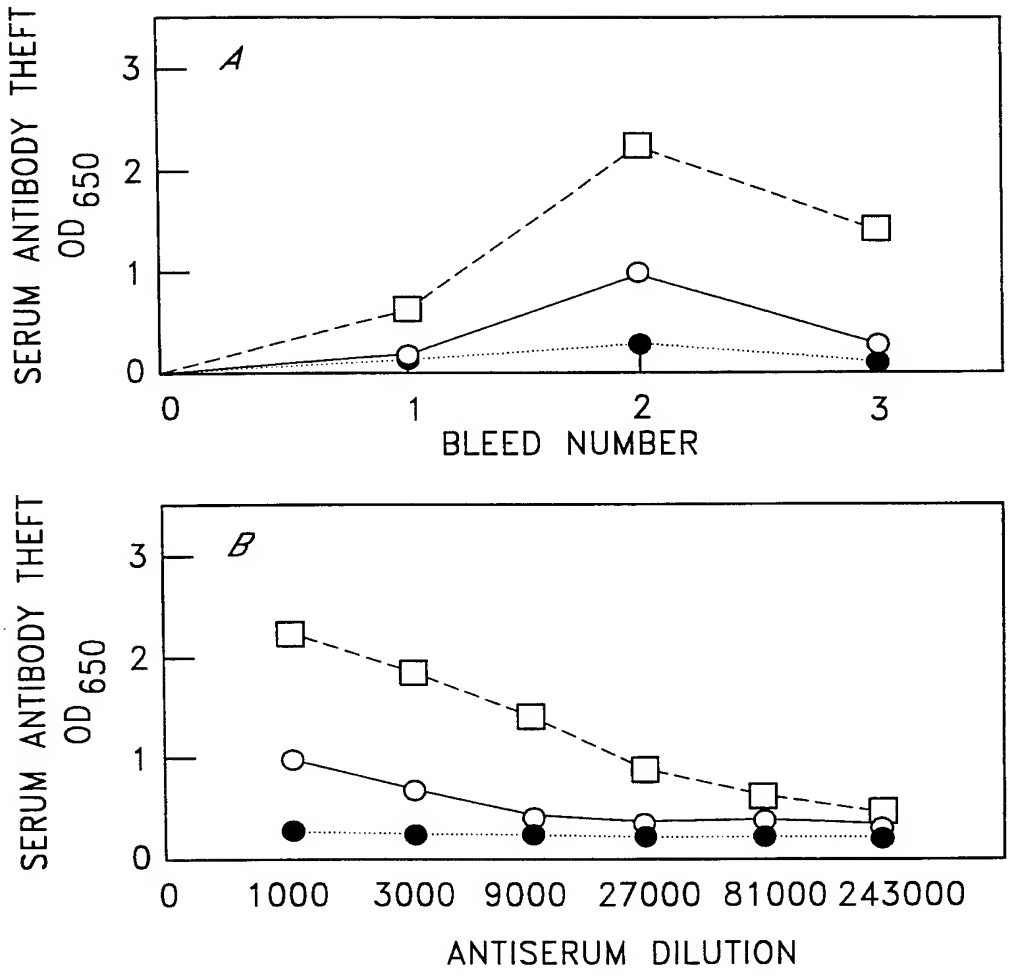

A
SERUM ANTIBODY THEFT
OD 650
3
2
1
0
0
1
2
3
BLEED NUMBER
B
SERUM ANTIBODY THEFT
OD 650
3
2
1
0
0
1000
3000
9000
27000
81000
243000
ANTISERUM DILUTION

FIG. 10

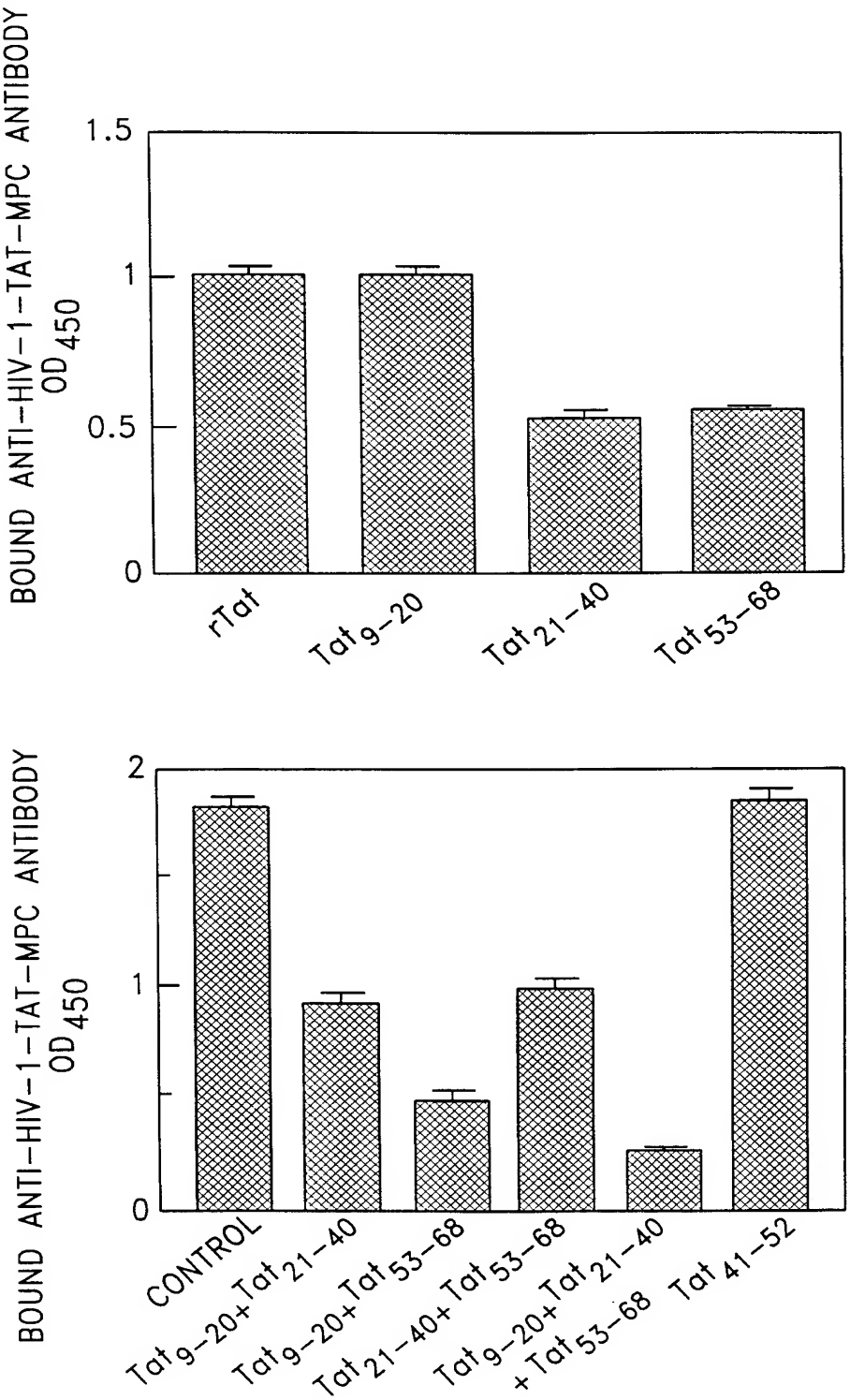

BOUND ANTI-HIV-1-TAT-MPC ANTIBODY OD450
1.5
1
0.5
0
rTat
Tat9-20
Tat21-40
Tat53-68
BOUND ANTI-HIV-1-TAT-MPC ANTIBODY OD450
2
1
0
CONTROL
Tat9-20+Tat21-40
Tat9-20+Tat53-68
Tat21-40+Tat53-68
Tat9-20+Tat21-40 +Tat53-68
Tat41-52

FIG. 11

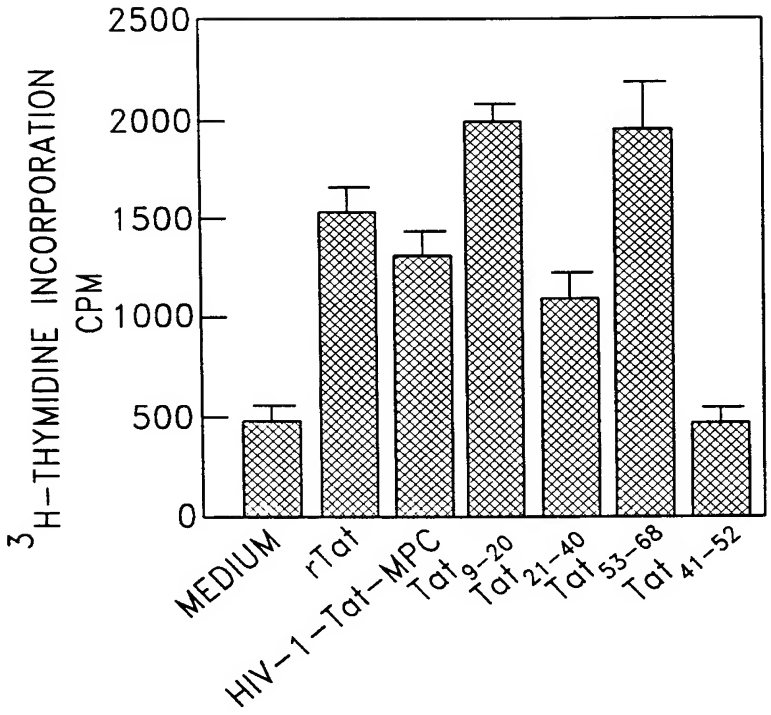

2500
2000
1500
1000
500
0
^{3}H-THYMIDINE INCORPORATION
CPM
MEDIUM
rTat
HIV-1-Tat-MPC
Tat 9-20
Tat 21-40
Tat 53-68
Tat 41-52

FIG. 12

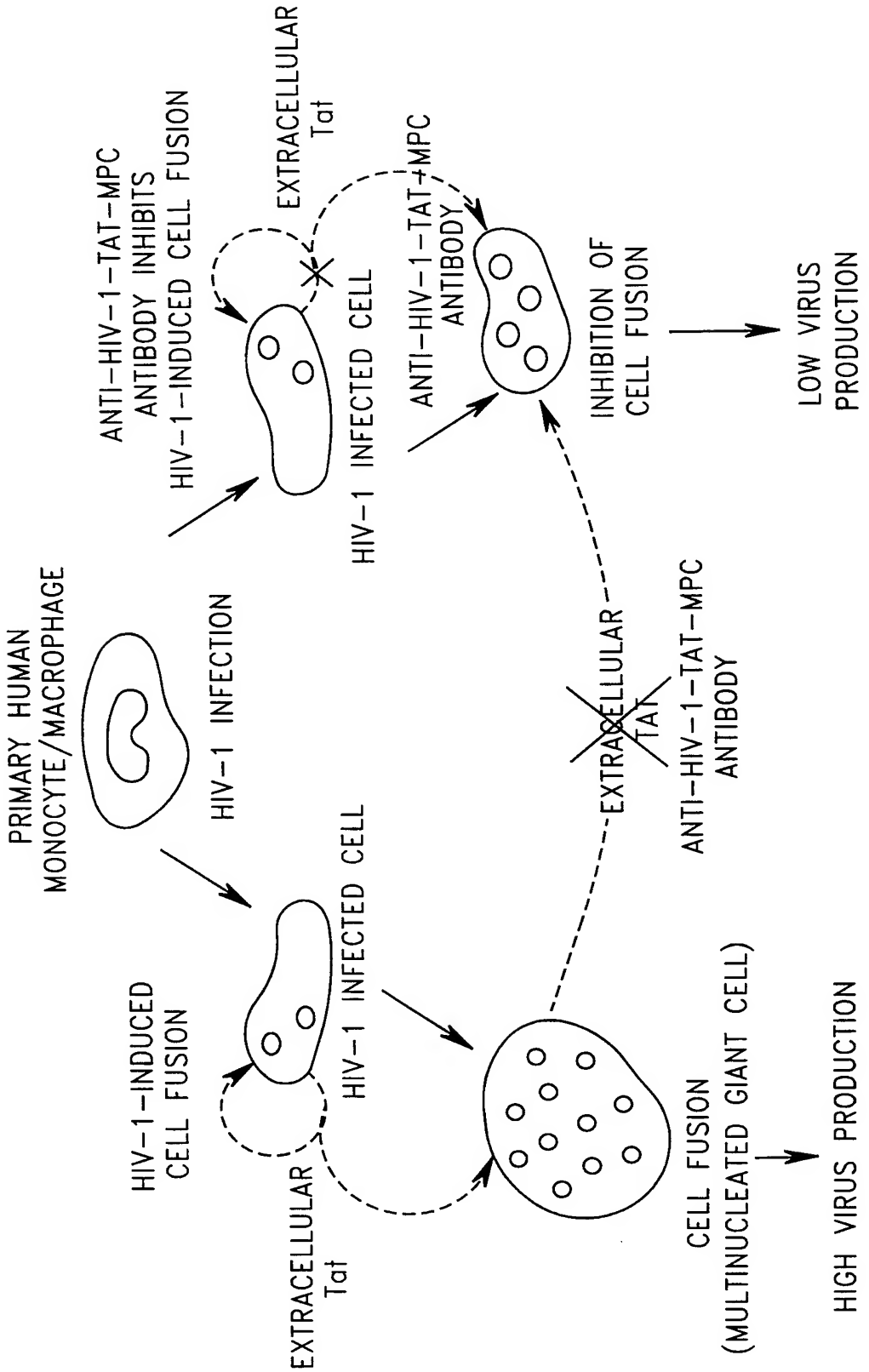

PRIMARY HUMAN MONOCYTE/MACROPHAGE
HIV-1 INFECTION
HIV-1-INDUCED CELL FUSION
HIV-1 INFECTED CELL
EXTRACELLULAR Tat
ANTI-HIV-1-TAT-MPC ANTIBODY INHIBITS HIV-1-INDUCED CELL FUSION
EXTRACELLULAR Tat
HIV-1 INFECTED CELL
ANTI-HIV-1-TAT+MPC ANTIBODY
INHIBITION OF CELL FUSION
LOW VIRUS PRODUCTION
EXTRACELLULAR TAT
ANTI-HIV-1-TAT-MPC ANTIBODY
CELL FUSION (MULTINUCLEATED GIANT CELL)
HIGH VIRUS PRODUCTION

FIG. 13

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 00/10119

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/49 C07K14/16 A61K38/10 A61K39/02 C07K5/037
C07K14/445

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 02185 A (THYMON L L C ;GOLDSTEIN GIDEON (US)) 21 January 1999 (1999-01-21) The whole document; see especially page 23 and Examples 1,2,4	1-10,38, 40,44, 45,60-62
X	WO 99 27958 A (ENSOLI BARBARA ;IST SUPERIORE SANITA (IT)) 10 June 1999 (1999-06-10) claims 11,46; example 1; table 2	1-10
X	WO 94 15634 A (RATH MATTHIAS) 21 July 1994 (1994-07-21) See especially SEQ ID NOS 6 and 8	1-9

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	15. 11. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 22349 A (INST HUMAN GENETICS BIOCHEM ;RODMAN TOBY C (US)) 11 November 1993 (1993-11-11) See especially Tables 1 and 4	1-10
X	WO 99 26656 A (INST HUMAN GENETICS BIOCHEM ;RODMAN TOBY C (US)) 3 June 1999 (1999-06-03) see especially Fig.5	1-10
X	ALBINI ADRIANA ET AL: "Identification of a novel domain of HIV Tat involved in monocyte chemotaxis." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 26, 26 June 1998 (1998-06-26), pages 15895-15900, XP000925779 ISSN: 0021-9258 abstract; figure 2	1-10
X	TAHTINEN M ET AL: "B-cell epitopes in HIV-1 Tat and Rev proteins colocalize with T-cell epitopes and with functional domains." BIOMEDICINE & PHARMACOTHERAPY, vol. 51, no. 10, December 1997 (1997-12), pages 480-487, XP000933416 ISSN: 0753-3322 abstract; table 1	1-10
X	HSIAU-WEN HUANG ET AL: "Structural characterization of the metal binding site in the cysteine-rich region of HIV-1 Tat protein" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 227, no. 2, 1996, pages 615-621, XP000929504 ORLANDO, FL US page 616	1-9
P,X	BOYKINS RA ET AL: "Cutting edge: A short polypeptide domain of HIV-1-Tat protein mediates pathogenesis" JOURNAL OF IMMUNOLOGY., vol. 163, 1 July 1999 (1999-07-01), pages 15-20, XP000929461 THE WILLIAMS AND WILKINS CO. BALTIMORE., US ISSN: 0022-1767	1-10
A	the whole document	38,40, 44,45, 61,62

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	AHLBORG N: "Synthesis of a diepitope multiple antigen peptide containing sequences from two malaria antigens using Fmoc chemistry" JOURNAL OF IMMUNOLOGICAL METHODS,NL,ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, vol. 179, no. 2, 27 February 1995 (1995-02-27), pages 269-275, XP004021093 ISSN: 0022-1759	11-13, 34,48, 60-62
Y	the whole document ---	14-23
Y	NARDIN E H ET AL: "Plasmodium falciparum polyoximes: highly immunogenic synthetic vaccines constructed by chemoselective ligation of repeat B-cell epitopes and a universal T-cell epitope of CS protein" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 6, 1 April 1998 (1998-04-01), pages 590-600, XP004110755 ISSN: 0264-410X the whole document ---	14-23
P,X	BOYKINS R A ET AL: "SYNTHESIS AND CONSTRUCTION OF A NOVEL MULTIPLE PEPTIDE CONJUGATE SYSTEM: STRATEGY FOR A SUBMIT VACCINE DESIGN" PEPTIDES,ELMSFORD,US, vol. 21, 2000, pages 9-17, XP000933414 ISSN: 0196-9781 the whole document ---	1-62
A	LU Y -A ET AL: "CHEMICALLY UNAMBIGUOUS PEPTIDE IMMUNOGEN: PREPARATION, ORIENTATION AND ANTIGENICITY OF PURIFIED PEPTIDE CONJUGATED TO THE MULTIPLE ANTIGEN PEPTIDE SYSTEM" MOLECULAR IMMUNOLOGY,US,ELMSFORD, NY, vol. 28, no. 6, 1 June 1991 (1991-06-01), pages 623-630, XP000579876 ISSN: 0161-5890 See Fig. 3 --- -/--	11-62

INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/US 00/10119

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	LE T P ET AL: "Immunogenicity of Plasmodium falciparum circumsporozoite protein multiple antigen peptide vaccine formulated with different adjuvants" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 2-3, 2 January 1998 (1998-01-02), pages 305-312, XP004098640 ISSN: 0264-410X figure 1 -----	11-62

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9,62 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 11-23,34,48-62(all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 11-23,34,48-62(all partially)

With respect to the multiple peptide conjugates defined by subject 4 the following is noted:
The claims 11-23 and 34 relate to general structures containing at least partly generic definitions of the substituents, which also can be partly absent. Moreover said claims are also defined by a desirable property, that is, the possibility of selective attachment of (antigenic) peptides. Therefore a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the complete subject-matter of said claims impossible.
Furthermore the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the concept of selective attachment to an amino acid residue of a multiple antigen construct having two reactive groups and to compounds defined in the claims 24-33, 35-47, their preparation and use.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 4,5; in part:1-3,6-10,40,45,60-62

Peptid comprising a portion of the Tat protein selected from the group consisting of the portion from amino acids 21-40 of the Tat protein and relating to SEQ ID NOs 1,5,8,11,14,17,20 and 22-66; method of reducing the acitivity of Tat in an individual infectd with HIV or inducing an immune response against the Tat protein. Multiple peptide conjugate, wherein at least one of the HIV peptides comprises a peptides selected form the group consisting of SEQ ID NOs 1,5,8,11,14,17,20 and method for making said multiple peptide conjugate. Use of said multiple peptide conjugate for inducing an immune response.

2. Claims: in part:1-3,6-10,40,45,60-62

Peptid comprising a portion of the Tat protein selected from the group consisting of the portion from amino acids 53-68 of the Tat protein and relating to SEQ ID NOs 2,6,9,12,15,18,21. Method of reducing the acitivity of Tat in an individual infectd with HIV or inducing an immune response against the Tat protein. Multiple peptide conjugate, wherein at least one of the HIV peptides comprises a peptides selected form the group consisting of SEQ ID NOs 2,6,9,12,15,18,21 and method for making said multiple peptide conjugate. Use of said multiple peptide conjugate for inducing an immune response.

3. Claims: in part:1-3,6-10,40,45,60-62

Peptid comprising a portion of the Tat protein selected from the group consisting of the portion from amino acids 9-20 of the Tat protein and relating to SEQ ID NOs 3,4,7,10,13,16,19; method of reducing the acitivity of Tat in an individual infectd with HIV or inducing an immune response against the Tat protein. Multiple peptide conjugate, wherein at least one of the HIV peptides comprises a peptides selected form the group consisting of SEQ ID NOs 3,4,7,10,13,16,19 and method for making said multiple peptide conjugate. Use of said multiple peptide conjugate for inducing an immune response.

4. Claims: 11-39,41-44,46-59; in part: 40,45,60-62

Multiple peptide conjugate and method for making it. Use of said multiple peptide conjugate for inducing an immune response.

INTERNATIONAL SEARCH REPORT

Information on patent family members

rnational Application No
PCT/US 00/10119

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9902185 A	21-01-1999	US 5891994 A	06-04-1999
		AU 8392798 A	08-02-1999
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